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Understanding the Role of Glucose-Sensor HEXOKINASE in Seedling Establishment in *Arabidopsis thaliana*

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, to any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own. Included publications are as much my own work as indicated in the “author contributions” section of the manuscript in Appendix A.

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Contents

i. Abstract

This study investigates the complex role of *HEXOKINASE* (*HXK*) genes, with a focus on *HXK1*, in *Arabidopsis thaliana* seedling development and glucose sensing mechanisms. HEXOKINASEs are a family of glucose-phosphorylating enzymes that facilitate the breakdown of glucose to produce energy. Through a series of experiments employing mutant lines and biochemical assays, we explore the multifaceted functions of HXK1 in regulating seedling growth. Initial experiments establish HXK1 as a key glucose response factor, demonstrating its role as a glucose sensor/signal. Mutant seedlings exhibit reduced sensitivity to high glucose concentrations, implicating HXK1 in glucose signaling. Further analyses reveal growth deficiencies in *HXK* family genetic mutants, particularly in dark and low-light environments, emphasizing the crucial role of HXK1-mediated signaling in hypocotyl elongation regulation. Genetic and biochemical approaches elucidate the dependence of HXK1-mediated growth on its enzymatic function, highlighting the interactions between signaling components and glucose metabolism. Additionally, mutants of enzymes downstream of HXK1 in glycolysis show phenotypic similarities, underscoring the significance of glycolysis in seedling development. Investigation into the signaling mechanisms underlying HXK1 function, particularly in response to exogenous glucose and glucose-6-phosphate (G6P), reveals that HXK1 signaling primarily operates under high-glucose conditions, indicating complex regulatory networks in seedling growth. Moreover, analysis of catalytically inactive *HXK1* alleles display unexpected findings, implicating glucose homeostasis in seedling development. Furthermore, this study explores the molecular basis of gene misregulation in the *gin2-1* mutant, highlighting the role of HXK1-mediated signaling pathways in plant growth and stress responses. Transcriptomic analyses uncover significant patterns of misregulation in *gin2-1*, suggesting a starvation-type response and diminished sugar catabolism capacity consistent with findings outlined in our investigation of the HXK1-mediated growth phenotype. Investigation into the intersection between HXK1, PHTOCHROME INTERACTING FACTORS (PIFs), and SNF-RELATED KINASE 1 (SnRK1) by analysing the patterns laid out in the transcriptomes of key mutant lines revealed overlap in misregulated genes, providing insights into cross-regulatory processes. Lastly, this study investigates the regulatory mechanisms between PHYTOCHROME INTERACTING FACTOR 7 (PIF7) and HXK1 under End of Day Far Red (EoDFR) conditions, revealing unexpected induction of *HXK* family genes in wild-type (Wt) seedlings and implicating PIF7 in G6P-mediated hypocotyl elongation. Examination of genes relevant to SnRK1 signaling in

EoDFR conditions corroborates trends outlined in our transcriptomic analysis and also suggest some form of novel interaction between PIF7 and SnRK1. The study provides insights into the regulatory networks governing plant growth and highlights potential interactions between sugar signaling pathways and light perception mechanisms mediated by PIF transcription factors, demonstrates PIF7 dependence on HXK1 in a previously undescribed manner, and begins to shape a model to lay a framework to further test the hypotheses laid out.

ii. Lay Summary

In a period of history defined by climate change, it is imperative that we investigate the inner workings of plant signaling networks in order to protect our food sources from an ever-changing environment. Our study investigates the role of sugar signaling in the model species *Arabidopsis thaliana* on seedling development; impaired seedling growth can result in diminishing yields, weaker immune systems, and importantly for this study adaptation to changes in light and temperature in their growth environment. We focus on analysing the role that HEXOKINASE1, an enzyme important in core glucose metabolism, plays in seedling development and how this molecule interacts with other signal pathways in order to influence important growth parameters. Critically, we are interested in the perception of red light and far-red light that defines the growth of plants in shade and how that is influenced by sugar signaling via HXK1. In this study, we use a variety of techniques to explore the nature of HXK1's role in seedling development, compare the transcriptomic profile of mutants lacking *HXK1* to mutants for other important signaling genes in order to understand the wider implications of HXK1 activity, and finally take a closer look at the interplay between shade detection and sugar signaling. Through these experiments, we seek to gain a better understanding of how these plants coordinate their growth through multiple signals, and this work will allow others to begin exploring these mechanisms in other species of plants more important to our food chain. More work is needed to fully understand the complex interactions laid out in this study, but we are excited at the possibility of greater understanding that may influence the wider scientific community, and through them the global food supply.

iii. Acknowledgements

I would like to thank everyone that made it possible to finish my PhD. To Professor Karen Halliday, whose endless patience and guidance I will be forever grateful for, especially during the trying times of the COVID-19 pandemic and the aftermath of lockdown. I would like to also thank the Darwin Trust of Edinburgh for their financial support throughout my PhD, and whose generosity cannot be overstated in their funding of my additional extension time needed to finish my project. To Prof. Steven Spoel, for being a critical eye in my thesis committee and ensuring that I would be able to explain my results in the face of scrutiny, while always maintaining a kind presence.

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Chapter 1. Introduction and Literature Review

1.1 General Introduction

Plant development is intimately tied with sugar resource availability; photosynthetically derived and environmental sugars (sugar present in the soil) and their abundance can dramatically affect the way that a plant grows. It comes as no surprise that because of its importance, plants need to be able to sense and respond to sugar in very intimate ways. Likewise, the perception of light is critically important for plants to respond to seasonality and regulating their growth. Through these and several other signals, plants can shift their growth, both spatially and temporally, in order to maximise their chances of surviving and thriving to produce further offspring. It stands to reason that all of these responses and signaling pathways that translate the perception of these external phenomena into genetic regulatory changes can connect and feed into each other in order to give the whole system the best chance at weighing all its options and growing in the most energy efficient ways.

1.2 Sugar Signaling

Numerous genes involved in sugar sensing and signaling have been identified through mutant screens that display altered responses to exogenous sugars during seed germination and early seedling growth in *Arabidopsis thaliana*. For instance, glucose-*insensitive* (*gin*) mutants fail to exhibit growth arrest when exposed to inhibitory high levels of glucose, displaying normal hypocotyl elongation, cotyledon greening, and expansion (Zhou et al, 1998; Eveland and Jackson, 2012). Independent screens for other sugar response phenotypes revealed that specific mutations were found to be allelic to *gin* loci, suggesting their potential roles at the intersection of different sugar signaling pathways (Eveland and Jackson, 2012).

Sugar-based signaling pathways interact with various hormones to regulate crucial aspects of plant growth. Generally, plants with defective abscisic acid (ABA) and/or ethylene perception and signaling tend to exhibit altered responses to sugar. Moreover, emerging connections between sugars and other plant hormones such as auxin and brassinosteroids indicate potential regulation of specific developmental processes (Eveland and Jackson, 2012).

Several master regulators of glucose-regulated signaling components modulate plant growth. These key components include glucose sensor HEXOKINASE1 (HXK), Trehalose 6-

Phosphate (T6P), a signaling molecule, and TARGET OF RAPAMYCIN (TOR) and SNF1-RELATED PROTEIN KINASE1 (SnRK1) kinases (Sheen, 2014; Li and Sheen, 2016). Total disruption of the SnRK and TOR complexes have been shown to be embryo lethal, thus requiring partial mutation or inducible knockouts to study their roles in various processes (Broeckx et al, 2016; Brunkard, 2020).

SnRK1 is a multiprotein complex that includes a kinase α subunit and two regulatory β and βγ subunits and is analogous to the well characterized AMP-ACTIVATED PROTEIN KINASE (AMPK) in animals and SNF1 in yeast which serve similar roles (Baena-González et al, 2007). SnRK1 activity is induced by sugar resource deprivation and is repressed by the sugars glucose-6-phosphate (G6P), glucose-1-phosphate, and trehalose-6-phosphate (T6P) (Nunes et al, 2013). The activation of the SnRK1 complex leads to the induction of catabolic adenosine triphosphate (ATP) producing processes and inhibition of various anabolic processes that consume ATP (Crozet et al, 2014), and this flux permits growth adjustments based on nutrient availability, which is intimately tied to photosynthetic output. SnRK1 works primarily through, as its name implies, protein phosphorylation of key metabolic enzymes to modify the active response to sugar and targeting various transcription factors to modify their activity (Pedrotti et al, 2018; Wurzinger et al, 2018)

In plants, TOR is a large multidomain serine/threonine kinase that interacts with REGULATORY-ASSOCIATED PROTEIN OF TOR (RAPTOR) and the LETHAL WITH SEC THIRTEEN 8 (LST8) proteins to form the TOR COMPLEX 1 (TORC1) (Shi et al, 2018). Like its counterpart SnRK1, TOR is an evolutionarily conserved kinase that plays central roles in plants, animals, and yeast. Plants only have a single *TOR* gene responsible for the TOR kinase function, which regulate a vast array of biological responses to link growth, stress resistance, translation, and more (Deprost et al, 2007). TORC1 is activated by sugars and amino acids, as well as the phytohormone auxin (Schepetilnikov et al, 2013; Dobrenel et al, 2016). TORC1 phosphorylates subsequent kinases like the ribosomal protein S6 kinase (S6K), known to positively regulate TOR activity (Schepetilnikov et al, 2013). Another kinase, YET ANOTHER KINASE 1 (YAK1), has recently gained attention as a positive regulator of TOR activity while being simultaneously regulated by TOR (Forzani et al, 2019). Opposite to SnRK1, the activation of TORC1 triggers the initiation of anabolic processes and halts catabolic processes (Dobrenel et al, 2016). Furthermore, TORC1 activity stimulates ribogenesis, translation, and cell-cycle advancement, linking TORC1 to growth-stimulating processes particularly in meristem regions, and mutations in TOR partners result in

compromised plant developmental processes, including flowering, shoot development, and root growth (Xiong et al, 2013; McCready et al, 2020).

1.2.1 HXK1 Signaling

Among sugar response regulators, HXK1 plays a central role in glucose sensing and plant growth. HXK1 irreversibly phosphorylates glucose, initiating its incorporation into the glycolytic pathway. HXK1 has been suggested to function as a sugar sensor and a sugar-signaling component in nuclear transcriptional regulation (Jang and Sheen, 1997). In addition to its conserved enzymatic role in glycolysis, HXK1 has been shown to be involved in regulating glucose sensitivity and photosynthetic gene repression in response to glucose (Moore et al., 2003). Notably, the *gin2-1* mutant, with a missense mutation in the *Arabidopsis HXK1* locus, was identified as a glucose sensor in seedlings. This mutant displayed normal seedling development under physiologically toxic levels of glucose, and further investigation with two catalytically inactive alleles with conserved signaling activity (two separate point mutations: serine 177 to alanine [S177A] and glycine 104 to aspartic acid [G104D] that inhibit the phosphorylation of glucose by HXK1) expressed in mutant backgrounds showed wild-type (Wt) development, thus decoupling the catalytic and signaling roles of HXK1 into molecularly distinct functions (Moore et al., 2003).

HXK1 has been shown to have dual roles in glycolysis and nuclear signaling, with the ability to regulate gene expression in response to glucose. It interacts with partner proteins VACUOLAR ATP SYNTHASE SUBUNIT B1 (VHA-B1/VAB1) and REGULATORY PARTICLE TRIPLE-A ATPASE 5B (RPT5B) and is essential for glucose-induced gene repression (Cho et al., 2007). Glucose-induced repression of photosynthetic genes, such as CAB2 and CAA, is attributed to a negative feedback mechanism mediated by HXK1 (Cho et al., 2006). However, the exact mechanisms of how sugar facilitates HXK1 nuclear localization or complex assembly at the chromatin remain unclear; contradictory findings exist regarding the nuclear localization of HXK1, as some studies were unable to detect GFP-tagged HXK1 in the nuclear fraction of leaf tissue (Balasubramanium et al., 2007), while others confirmed its presence in the nucleus in protoplasts (Cho et al., 2006).

Though this study is primarily concerned with seedling development, it is worth noting that HXK1 has a significant role in leaf development; a recent study demonstrated that both cell division and expansion were regulated by HXK1 during early leaf development, and that *hxk1-3* mutant plans show reduced sensitivity to sucrose-induced division in leaves (Van

Dingenen et al, 2019). As such, we can see that HXK1 represents an important step in plant development at multiple life stages, so uncovering the nature of how it signals and interacts with other mechanisms is of great importance to the plant biology community. As HEXOKINASEs are highly conserved across a large range of species outside of the plant kingdom, such as humans and yeast, these findings may prove useful to other fields as well (Rodríguez-Saavedra et al, 2021).

1.2.2 Trehalose Signaling

Trehalose, a non-reducing disaccharide, possesses notable attributes such as high solubility and chemical stability which enable it to harmonize with cellular metabolic processes, even at elevated concentrations. This distinctive quality positions trehalose as the preferred compound in various organisms, including bacteria, fungi, and invertebrates. Within these organisms, trehalose serves multiple functions, including osmoregulation, stress mitigation, and serving as a medium for carbon storage and transport (Elbein, 1974; Bonini et al., 2004).

In contrast, vascular plants primarily rely on sucrose, another non-reducing disaccharide, to perform many of these vital roles (Lunn, 2008). Typically, sucrose vastly outweighs trehalose in higher plants, with concentrations ranging from 100 to 1000 times higher (Carillo et al., 2013). The flow of newly assimilated carbon into sucrose significantly exceeds the flow into trehalose, differing by approximately four orders of magnitude in the case of *Arabidopsis* rosettes (Szecowka et al., 2013). The prevalence of sucrose in plant physiology may be attributed to its superior transport properties, enabling it to effectively meet the diverse metabolic demands of vascular plants.

However, trehalose metabolism is still a critical part of sugar sensing and growth regulation in vascular plants; trehalose-6-phosphate (T6P) accumulation is directly tied to sucrose status, and application of exogenous sucrose results in rapid accumulation of T6P in seedlings at consistent ratios across studies, and that sucrose is correlated most strongly with T6P accumulation over other sugars and sugar analogues (Lunn et al, 2006, Yadavet al, 2014). T6P has also been shown to be essential to plant development, where mutants lacking *TREHALOSE-6-PHOSPHATE SYNTHASE 1* (*TPS1*) are embryo lethal; in order to study these genes effectively, partial knockout mutations are used (Eastmond et al, 2002). TPS1 appears to be the only catalytically active form of TPS in *Arabidopsis*, with several family members that appear to be unable to catabolise G6P or UDP-glucose into T6P (Vandesteene et al, 2010). T6P acts as an inhibitor to SnRK1, binding to KIN10 and inhibiting its function

in young *Arabidopsis* tissues by a yet undescribed mechanism, though the fractions of KIN10 that T6P bind to have been described (Zhang et al, 2009; Nunes et al, 2013).

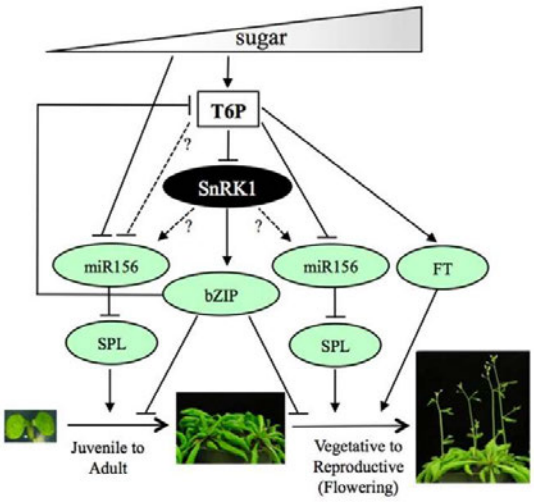

Fig. 1.1 T6P and SnRK1 Network. Figure from Tsai and Gazzarrini, 2014. This figure demonstrates the role of SnRK1 in regulating multiple growth nodes through different pathways, and how trehalose-6-phosphate (T6P) accumulation regulates SnRK1 and vice-versa.

SnRK1 is a master regulator gene, responsible for major changes in growth architecture and is the core of metabolic homeostasis in plant (Emmanuelle et al, 2016). Given its importance in development, it becomes relevant to study the impact of G6P and T6P on growth patterning in the seedling, something that we will explore in detail in the course of this study.

1.3 Light Signaling

As sessile photoautotrophic organisms, plants must adeptly perceive and respond to their ambient light environment to optimize growth, development, and overall survival (Neff et al., 2000). Light serves a dual function in the life of plants: it is the fundamental energy source for photosynthesis and concurrently acts as a primary environmental signal, governing a broad spectrum of physiological and morphological processes, ranging from germination to flowering (Li et al., 2012). Notably, light not only serves as the pivotal substrate for photosynthesis, enabling the synthesis of crucial carbohydrates, but also intricately mediates various developmental phenomena. These include the orchestration of seed germination, the establishment of seedlings, the activation of meristems, the expansion of leaves, the elongation of stems, the phenomenon of phototropism, neighbour detection, the regulation of circadian rhythms, and the precise timing of flowering (Li et al., 2012; Roig-Villanova et al., 2006).

This dynamic adaptation of developmental processes to the qualities and quantities of light is strikingly manifested during the transition of seedlings from their subterranean existence to the aboveground realm. When seedlings germinate in the light-deprived soil environment, they adopt an etiolated growth pattern, characterized by a notably elongated hypocotyl, underdeveloped cotyledons, and a rudimentary root system. This phenomenon, known as skotomorphogenesis, serves the primary objective of elongating rapidly to reach potential sunlight sources. However, upon breaching the soil surface and encountering sunlight, the growth of the hypocotyl ceases, the cotyledons undergo expansion to initiate photosynthesis, and the root and shoot apical meristem populations are activated, heralding the switch to photomorphogenesis (Arsovski et al., 2012; Nemhauser, 2008). Photoreceptors in plants play an instrumental role in curbing hypocotyl elongation during photomorphogenesis, necessitating a comprehensive reprogramming of the *Arabidopsis* transcriptome to facilitate this transition (Ma et al., 2001). Plants encounter stress in response to both high and low light intensities, and thus, both conditions pose unique challenges. High light intensities can induce oxidative stress, leading to photodamage, while low light intensities can limit photosynthetic capacity, ultimately leading to energy and nutrient deficits (Fankhauser & Batschauer, 2016). It is imperative for plants to display remarkable plasticity in their responses to these varying light conditions, such as the adjustment of leaf and chloroplast positions to minimize exposure or enhance light capture for efficient photosynthesis (Fankhauser and Batschauer, 2016).

1.3.1 Red Light Photoreceptors

Phytochromes (PHYs) are a group of light-sensitive receptors first identified in plants back in 1959 and exhibit a remarkable ubiquity across the biological spectrum (Butler et al., 1959). Their presence extends beyond the plant kingdom, encompassing specific fungi and numerous prokaryotic organisms (Burgie et al., 2014). These photoreceptors possess a distinctive capacity to assess the proportion of red light (R; 600-700 nm) to far-red light (FR; 700-800 nm), thereby endowing plants with the ability to perceive and adjust to variations in photosynthetically active radiation (PAR; 400-700 nm) originating from adjacent plant life (Rausenberger et al., 2010; Roig-Villanova & Martínez-García, 2016).

PHYTOCHROMES exist as either homodimeric or heterodimeric soluble phosphoproteins. In *Arabidopsis thaliana*, these phytochromes are encoded by five genes (phyA-phyE) (Rockwell et al., 2006). Each of these photoreceptors plays diverse roles, sharing some functionalities while also undertaking distinct responsibilities in processes such as seed

germination, de-etiolation, floral transition, and responses related to shade avoidance (Chory, 1991; Kami et al., 2010; Leivar & Monte, 2014; Chen et al., 2014; Sakuraba et al., 2014). A significant characteristic of phytochromes in *Arabidopsis thaliana* classifies them into two types: type I, characterized by their light-lability featuring phyA as the sole representative, and type II phytochromes, stable in the presence of light (phyB to phyE) (Li et al., 2015).

PhyA, activated by FR light and suppressed by R light (Shinomura et al., 2000), fulfils a distinctive role in low-light conditions, distinguishing itself from type II phytochromes. It takes a central role in very low fluence responses (VLFR < 1μmol m-2 s-1) and the FR-high irradiance responses (FR-HIRs), regulating growth-related processes such as seed germination, de-etiolation, and day-length sensing (Casal, 2000; Kneissl et al., 2009; Staneloni et al., 2009). Conversely, the light-stable type II phytochromes (phyB-phyE) are responsible for suppressing the shade avoidance syndrome (SAS) signaling under sunlight, with phyB playing a predominant role (Franklin & Quail, 2010). The low fluence response (LFR 10–1000μmol m-2 s-1) of type II photoreceptors is characterized by its red (R)/far-red (FR) reversibility and dependence on red light for induction (Li & Hiltbrunner, 2021; Viczián et al., 2017).

Each phytochrome molecule consists of an N-terminal domain (~70 kDa) and a C-terminal domain (~55 kDa) linked by a flexible hinge region. These homodimers are synthesized in the cytosol and integrate a linear tetrapyrrole chromophore, phytochromobilin, which absorbs in the red-light spectrum in its inactive form (Pr), peaking at 660 nm (Chen & Chory, 2011). Upon exposure to light, phytochromes undergo a transformation into the biologically active Pfr form, with a peak at 730 nm in the FR light spectrum, and translocate to the nucleus (Chen & Chory, 2011; Viczián et al., 2017).

Intriguingly, phytochromes, owing to their intrinsic thermal instability, can undergo a light-independent relaxation process known as dark reversion/thermal reversion, returning to the inactive Pr state (Rockwell et al., 2006). This photoreversible interconversion, along with the presence of both stable forms in equilibrium under light conditions, empowers phytochromes to function as molecular switches. They initiate distinct biological responses based on the red/far-red (R/FR) ratio in the light environment (Deng & Quail, 1999). The phosphorylation status of specific serine residues in the N-terminal domain appears to play a pivotal role in modulating thermal reversion (Viczián et al., 2020). Furthermore, phytochromes, particularly phyB, play a vital role in temperature sensing, with elevated temperatures accelerating the

rate of phyB dark reversion. This renders them indispensable components for adapting to alterations in both light and temperature (Fig. 1.2) (Jung et al., 2016; Legris et al., 2016).

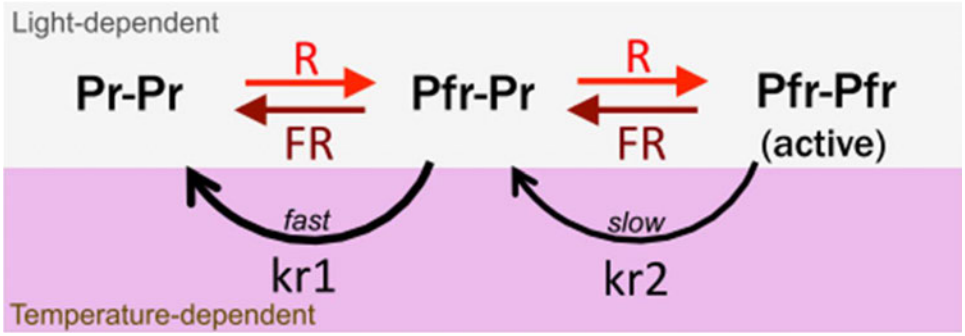

Figure 1.2 Phytochrome dimer red light and dark reversion. Figure from Halliday and Davis, 2016. Demonstrates the response of PHYTOCHROME protein dimer in exposure to both light and temperature from the active Pfr to inactive Pr form. R=red light. FR=far-red light. kr=Thermal reversion rate.

1.3.2 PHYTOCHROME INTERACTING FACTOR bHLH Transcription Factors

The PHYTOCHROME INTERACTING FACTOR (PIF) family of proteins have been one of the most well studied signals in plant photobiology, and act as a strong coordinator of growth (Castillon et al, 2007). The PIF family consists of basic helix-loop-helix (bHLH) transcription factors, named for the domain that is critical for binding to DNA and dimerization with other PIFs to initiate signaling, either through homodimerization or heterodimerization (Toledo-Ortiz et al, 2003; Bu et al, 2011). Currently there are seven PIF isomers (PIF1, PIF3, PIF4, PIF5, PIF7, and PIF8) that are able to interact with the active Pfr form of phytochrome B through a specialised active phyB-binding (APB) domain and are thus transported into the nucleus in subnuclear photobodies, colloquially known as “speckles.” (Huq and Quail, 2002; Huq et al, 2003; Leivar et al, 2012; Leivar and Monte, 2015). PIF1 and PIF3 also have an active phyA-binding (APA) domain that allows them to uniquely complex with phytochrome A (Shen et al, 2008). Upon their interaction with the PIFs, PHY proteins target PIFs for proteasomal degradation within these speckles; PIFs reciprocally regulate the size and number of these speckles which are involved in phyB-mediated signaling and facilitate phyB binding with CONSTITUTIVE PHOTOMORPHOGENESIS PROTEIN 1 (COP1), an E3 ubiquitin ligase protein that fascilitates the degredation of photoactive phyB (Chen et al, 2003; Chem, 2008; Al-Sady et al, 2006; Ni et al, 2017). During periods of darkness, the pool of photoactivated phytochrome will gradually revert thanks to the previously described thermal reversion process, and thus unable to bind to PIFs and target them for degradation,

allowing PIFs to begin accumulating and binding to DNA (Bauer et al, 2004; Monte et al, 2004; Shen et al, 2005; Shen et al, 2007).

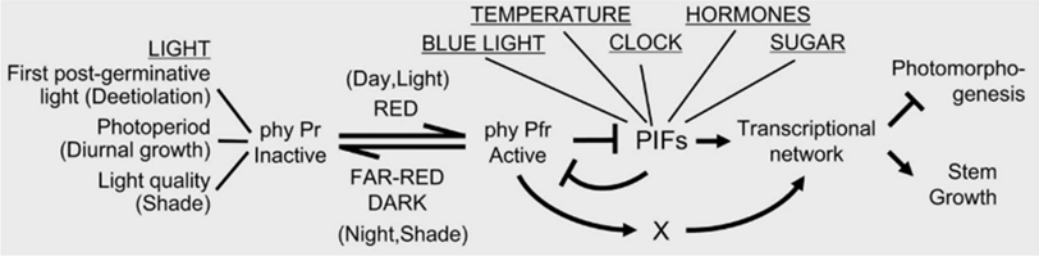

Figure 1.3 PIF acting as a central integrator of various signals. Figure from(Leivar & Monte, 2014). Figure demonstrates the multiple signal pathways influenced by PIFs and their regulation via the photoreversible PHYTOCHROMEs.

Curiously, while most PIFs are targeted for degradation in the light, PIF7 does not follow this pattern; when PIF7 interacts with photoactivated phyB, it accumulates rather than declines under conditions with enriched red light; under conditions with far-red light enrichment PIF7 is rapidly dephopshorylated by a currently undescribed phosphatase (Leivar et al, 2008; Li et al, 2012). It has been suggested that PIF7 moves between the cytoplasm and the nucleus depending on the phosphorylation status as determined by the red to far-red ratio and subsequent interactions with 14-3-3 scaffolding proteins (Huang et al, 2018). PIF7 also contains a unique long glutamine rich (poly-Q) sequence towards the N-terminus of the protein that it shares with PIF8, which can potentially result in additional protein-protein interactions or further transcriptional activity (Leivar et al, 2008; Freiman and Tijan, 2002). A recent study into the unique associations between PIF7 and PhyB confirm that this is at least in part due to the presence of the poly-Q domain in the protein, and that it facilitates condensate formation and is essential to its function in WL and shade (Xie et al, 2023). The unique nature of this PIF results in its seemingly dominant control over the far-red elongation response, which is particularly impactful when perceived at the end of the day (EoD) or the start of the night (SoN) in coordination with PIF4 and PIF5 (Mizuno et al, 2015).

When in an active state, the PIFs primarily function as persistent transcriptional (co-) activators for genes associated with dark- or shade-induced growth, including key players like *Arabidopsis thaliana HOMEOBOX PROTEIN 2 (ATHB2), PHYTOCHROME RAPIDLY REGULATED 1 (PRE1), YUCCA8, INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29), SMALL AUXIN UP RNA 19 (SAUR19),* and *GIBBERELLIC ACID INSENSITIVE (GAI)*. While some of these genes act as PIF activators, others serve as negative regulators of PIFs.

This establishes a delicate negative feedback loop, likely preventing excessive responses (Al-Sady et al., 2008; de Lucas et al., 2008; Hornitschek et al., 2009; Leivar et al., 2008; Oh et al., 2012; Huq et al., 2004). Additionally, besides their role as activators, PIFs can also function as constant transcriptional repressors of light-induced genes (Kidokoro, 2009; Lee & Thomashow, 2012; Oh et al., 2009, 2012; Toledo-Ortiz et al., 2010; Zhang et al., 2013).

It is worth noting that phytochromes are not the sole regulators of PIF-DNA binding. PIFs can interact with certain growth inhibitors such as the DELLA family of proteins (GAI, RGA, RGL1, RGL2, and RGL3) (Park et al., 2012). This interaction enables PIFs to integrate the light and gibberellin signaling pathways (Leivar & Monte, 2014). Gibberellins accumulate in darkness and induce the degradation of DELLAs via the 26S proteasome. However, during the daytime, GA synthesis slows down, allowing DELLAs to accumulate. DELLAs interact with PIFs through their bHLH binding motif, blocking the PIFs' DNA-binding capacity and consequently repressing hypocotyl growth (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolomé et al., 2010). Another example of a protein family capable of modulating PIF activity is the HLH proteins (HFR1, PAR1, etc.). These proteins possess a HLH structure but lack a basic domain, enabling them to form heterodimers with PIFs and impede their DNA-binding capacity (Fairchild et al., 2000; Hao et al., 2012; Hornitschek et al., 2009; Roig-Villanova et al., 2006). This PIF-HLH interaction establishes a negative feedback loop that finely tunes and regulates plant responses to light and shade, averting excessive elongation (Leivar & Quail, 2011).

A broad spectrum of work has unveiled a broader role for PIFs beyond their pivotal involvement in light signaling. They emerge as central signaling hubs, orchestrating diverse responses to environmental challenges, such as heat, drought, and starvation (Foreman et al., 2011; Franklin & Whitelam, 2007; Halliday et al., 2003; Halliday & Davis, 2016; Halliday & Whitelam, 2003; Johansson et al., 2014; Koini et al., 2009; Quint et al., 2016; Toledo-Ortiz et al., 2014). Interestingly for this study, it has recently been reported that PIF4 reciprocally regulates and is regulated by T6P-SnRK1 as well (Hwang et al, 2019). It is important to further understand the complex network that regulates PIF expression and activity as well as the factors that work concurrently with PIFs to regulate similar responses. This becomes of particular importance when considering the changing environment we find ourselves in; establishing importance and order of various factors in environmental responses allows us to engineer more flexible crops that can better acclimate to the changing climate of the 21st century.

Chapter 2: HXK1 Regulation of Seedling Development

2.1 Introduction

Successful germination and seedling establishment in *Arabidopsis* relies on the presence and utilization of sufficient reserves, including Triacylglycerols (TAG) and seed storage proteins (SSP), as well as minor sucrose amounts in the mature embryo cotyledons (Baud et al., 2008). Following dark germination, stored nutrients support hypocotyl elongation, prioritizing skotomorphogenesis over root and cotyledon development. This growth phase critically depends on two key enzymes, PHOSPHOENOLPYRUVATE CARBOXYKINASE 1 (PCK1) and ISOCITRATE LYASE (ICL), which catabolize fatty acids to produce sugars by gluconeogenesis and the glyoxylate cycle, respectively (Eastmond et al., 2000; Penfield et al., 2004). Skotomorphogenic metabolism is also regulated by multiple signaling components, including circadian-influenced factors such as *TIMING OF CAB EXPRESSION 1* (*TOC1*) and dark-specific metabolic factors, such as *STARCH-EXCESS 4* (*SEX4*), which result in dark-mediated changes in growth and development that lead to elongated seedlings with small cotyledons (Chaiwanon et al., 2016; Seluzicki et al., 2017). These metabolic changes have implications for how the plant will grow, even upon the perception of light and switch to the photomorphogenic program, characterised by repression of growth and cotyledon expansion. It is then important to understand the mechanisms of metabolism-associated genes and how they impact growth in order to fully comprehend the ways that these signals propagate throughout the seedling. To that end, we look towards the well-studied metabolic factor gene *HEXOKINASE 1* (*HXK1*).

When considering the storage of carbon resources in oilseed plants such as *Arabidopsis*, it is important to note that the primary forms of storage are TAGs and SSPs. These two components make up a significant 30-40% of the seed dry weight (Baud et al., 2008). As germination takes place, TAGs are converted into sucrose, which then acts as the primary source of energy for seedling development. This conversion process has been studied in depth, as evidenced by the research conducted by Eastmond et al. in 2000 and Cai et al. in 2020.

In sink tissues (shoots, roots, etc), sucrose is cleaved in different ways, depending on the specific enzyme involved. SUCROSE SYNTHASE is responsible for producing UDP-

glucose and fructose, whereas invertase cleaves the sucrose molecule to liberate glucose and fructose (Yoon et al., 2021). Once these hexose sugars are produced, a crucial step in their metabolism involves phosphorylation. This process is mediated by two enzymes: HEXOKINASE (HXK) and FRUCTOKINASE (FRK). HXK can phosphorylate both glucose and fructose, whereas FRK primarily phosphorylates fructose. The role of these enzymes in the metabolic pathways of these sugars has been extensively studied and is a vital area of research in plant biology.

HXK1 serves as a primary glucose receptor, playing dual roles as a mitochondrial-bound enzyme and a nuclear signaling factor (Cárdinas et al., 1998). The enzymatic function of HXK1 involves catalysing glucose phosphorylation to generate glucose-6-phosphate (G6P), which is crucial for providing energy in plant growth and metabolic maintenance. Additionally, HXK1 acts as a negative feedback inhibitor of photosynthetic genes when complexed with partner proteins RPT5B and VHAB1 in the presence of exogenous glucose (Cho et al., 2007). Notably, HXK1 is enriched in the *CAB2* promoter region, which contains characterized cis elements, including a G box that is also bound by red-light regulated LONG HYPOCOTYL 5 (HY5) (Maxwell et al., 2003).

Studies involving HXK1 mutants that uncouple the enzymatic and signaling roles reveal that the signaling function of HXK1 is important for seedling development, particularly during nutrient-limited conditions like darkness or low light (Moore et al., 2003). The *gin2-1* mutant, insensitive to elevated glucose, displays conditional phenotypes including reduced germination inhibition with glucose and small seedling stature under low light on sugar-enriched media. Notably, the catalytically inactive alleles *hxk1*^{S177A} and *hxk1*^{G104D} can rescue impaired phenotypes in the *gin2-1* mutant, emphasizing the critical requirement of HXK1 signaling function for seedling establishment.

Very little work has been done on other members of the *HXK* gene family, and their role in development is poorly characterised in *Arabidopsis thaliana*. *HXK1* is the pre-eminent member of the family in *Arabidopsis*, with strong orthology to other important glucokinases in yeast (*HXK2*) and humans/animals (*GLK*) (Jang et al, 1997). A benchmark study from 2008 characterizes the other family members in *Arabidopsis* based on their homology to *HXK1*; *HXK2* has the strongest identity with *HXK1*, is ubiquitously expressed in plant tissues, and maintains near *HXK1* levels of glucokinase activity (Karve et al, 2008). The same study notes that *HXK3* is approximately half as active as *HXK1*, expressed in multiple tissue types,

but is localised to the chloroplast almost exclusively. A later study reassesses this localisation for *HXK3* (called *pHXK* in this study for its plastid localisation) and demonstrates that it has an important role in mediating the sugar-based repression of photosynthetic gene expression in the plastid genome via *PGE* signal regulation (Zhang et al, 2010). Neither of these studies, however, detail the effects that these genes have on the elongation of seedlings.

A small sub-family of *HXK* genes are the *HEXOKINASE*-LIKE (*HKL*) genes, which maintain identity and glucose-binding functions akin to *HXK1* but are unable to phosphorylate glucose or other sugars (Karve et al, 2008). *HKL1* and *HKL2* are expressed in similar quantities to *HXK1* and in all tissue types, but *HKL3* is exclusively expressed in flower tissues, per the same study, and may not even bind to glucose due to significant indel site changes compared to other members of the family. Of these three genes, only *HKL1* appears to be studied further in later works. HKL1 acts as a positive effector of several glucose and ethylene interactions, indicating that it acts as a signal transducer between the two pathways, and has a minor role in repressing hypocotyl elongation in seedlings (Karve and Moore, 2009; Karve et al, 2012). With this relatively small amount of information available for most members of the HXK gene family, there is a large gap in our understanding of how these genes potentially co-regulate HXK1-mediated processes or even act alone on others.

The research in this chapter highlights the role of the glucose sensor HXK1 in nutrient-limited conditions. These findings shed light on the importance of HXK1 in the early stages of *Arabidopsis* growth and development, providing valuable insights into the role of glucose sensing and signaling in plants. Furthermore, we explore the importance of other members of the HXK gene family in the G6P-mediated growth response, and touch on the phenotypic similarities between PIF and HXK1 mutant seedlings. Akin to HXK1, red-light signalling exerts strong control over hypocotyl development (Leivar et al, 2008, Park et al, 2012, Park et al, 2018, Lilley et al, 2012). Red light is primarily perceived by PHYTOCHROME (PHY), which directly modifies the activity of PHYTOCHROME INTERACTING FACTORS (PIFs). And as there is strong phenotypic similarity between etiolated hypocotyls with impaired red-light perception and sugar signaling mutants, we were interested in the levels of potential co-regulation through these two pathways.

2.2 HEXOKINASE Effect on Growth in Seedlings

HXK1 has been recognised to be a key glucose response factor through a variety of studies (Sheen et al, 1999, Moore et al, 2003), and its activity as a sensor is evident when growing mutants under steadily increasing amounts of glucose (Fig. 1a).

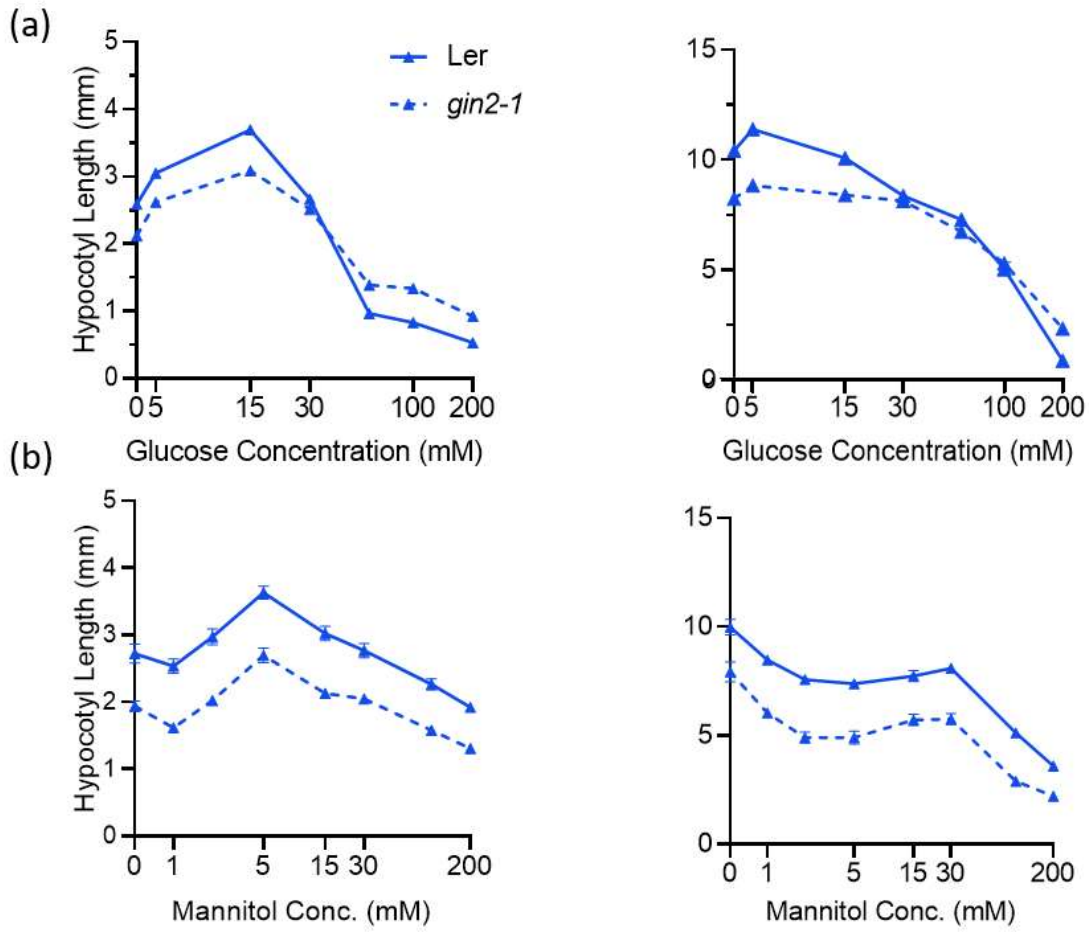

Fig 2.1 *gin2-1* Response to Exogenous Glucose and Mannitol. Seedlings grown in constant low light (5 µmol $m^{-2}s^{-1}$, left) and darkness (right) for 4 days on 0.5x MS medium. Seedlings supplemented with increasing amounts of (a) glucose to observe the response or (b) mannitol as a control for osmotic pressure. Error bars represent standard deviation between samples in three biological replicates. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant

As the amount of glucose in the growth medium increased, in both etiolated and constant low light (5 µmol $m^{-2}s^{-1}$) grown seedlings, there was a shift in the response at higher doses. In Wt seedlings, low doses of glucose promote growth, followed by an inhibition phase. *gin2-1*

mutants are shorter than Wt but show similar growth in the promotion phase and reduced sensitivity to glucose in the inhibition phase. Furthermore, using the osmotic control molecule mannitol, one can observe that an altered response is specific to the molecule and not due to an altered response to increased osmotic pressure by introducing foreign substances into the medium (Fig. 1b). These results align with previous reports of reduced sensitivity to high doses of glucose in *hxk1* mutant lines and furthers the model of HXK1 acting as a glucose sensor in developing seedlings; *gin2-1* seedlings have been shown to have 50% reduced glucose phosphorylation activity (Moore et al, 2003).

2.3 HEXOKINASE Mutants Exhibit Growth Deficiency in Dark and Low-Light Environments

gin2-1 mutant seedlings have documented short hypocotyls in a variety of different growth conditions, particularly in lower levels of light (Moore et al, 2003). PHYTOCHROMEs (PHY), red light photoreceptors, and the PHYTOCHROME INTERACTING FACTOR (PIF) family of proteins play a pivotal role in controlling growth in darkness and in low light, discussed in more detail in the literature review section of the thesis. In short, PIF transcription factors promote hypocotyl elongation when unperturbed by PHYs, which when activated by red light prevent PIF transcription activity by targeting them for degradation (Cheng et al, 2021). As such, we wanted to observe how *gin2-1* and *hxk1-3* respond to incrementally increasing the fluence of light (Figure 2a).

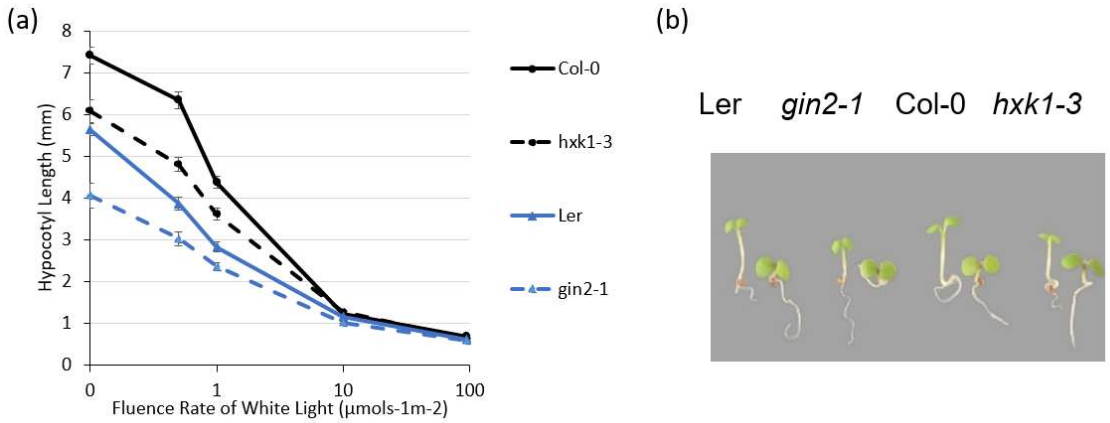

Fig. 2.2 HXK1 Regulates Skotomorphogenesis. (a) Wt (solid lines) and mutant (dashed lines) seedlings grown in constant light of increasing intensity. Colours assigned to relevant ecotypes; blue to *Landsberg erecta*, black to *Columbia-0* (b) representative seedlings of Wt and mutant grown in constant low (≥1 µmol $m^{-2}s^{-1}$, left) or higher (100 µmol $m^{-2}s^{-1}$, right) white light. All seedlings are 4 days old, grown on 0.5x MS media. Error bars represent standard deviation between samples in three biological replicates. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant in Ler. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0

Here, we observed that the short hypocotyl phenotype of the mutant seedlings was most apparent in darkness and extremely low fluence of light (≥1 µmol $m^{-2}s^{-1}$), whereas dim and moderately high levels of 95-100 results in hypocotyls almost indistinguishable from their Wt counterparts. Photographs of seedlings grown under low and moderately high levels of light demonstrated the difference in growth rate between these conditions (Figure 2b). These observations are consistent with the published literature, and this growth deficiency has been ascribed to the loss of the signalling capacity of HXK1 in these mutants (Moore et al, 2003). This experiment highlights the importance of light on the impact of HXK1-mediated growth, an important factor we recognised when furthering our investigation later in this chapter. In this respect, the phenotype is similar to mutant plants lacking *PIFs*, an important coincidence that will be explored in detail further in this chapter, as well as in the following chapters of this thesis. When giving closer examination to the way that HXK1 regulates hypocotyl growth, we wanted to independently test the signaling hypothesis of HXK1 mediated growth by observing the response of mutant and wild-type plants to the enzymatic product of HXK1 activity, G6P (Fig. 2.3)

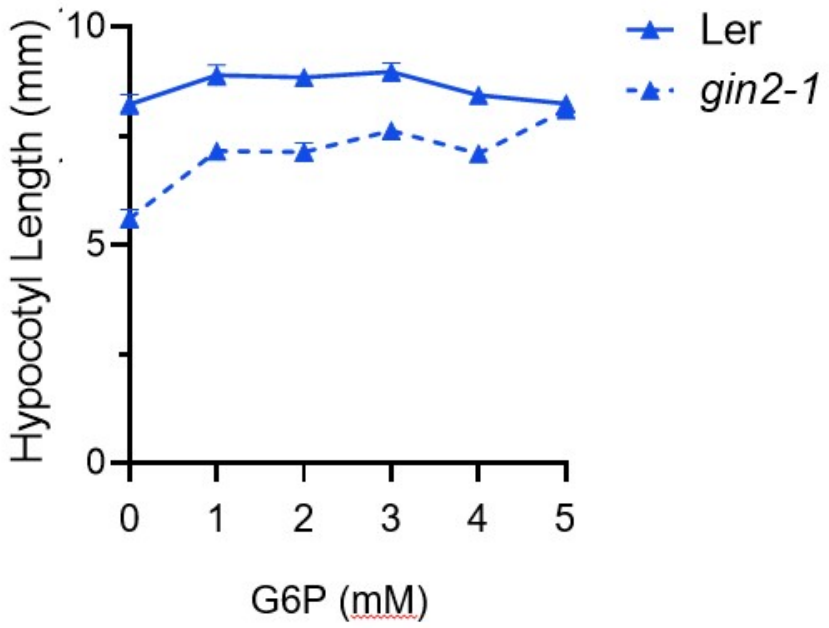

Fig. 2.3 G6P Complements *gin2-1* Short Hypocotyl Phenotype. Etiolated 4-day old seedlings grown on increasing amounts of G6P-supplemented 0.5x MS growth medium. Error bars represent standard deviation between samples in three biological replicates. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant in Ler.

What we observed was that G6P supplementation as little as 5mM was sufficient to rescue the short hypocotyl of both *gin2-1-1* and *hxk1-3* mutant plants, while showing little to no response in the wild-type. This is distinct from the response to glucose at increasing doses, as shown in Figure 2.1, where there is a delayed response. Here, we observed a consistent response unique to the mutants, suggesting that the growth defect of the mutant is a result of diminished G6P production; in other words, HXK1-mediated hypocotyl growth is facilitated by its role as an enzyme rather than a signalling molecule. This data appears to be at odds with published data, which indicates that the opposite is true (Moote et al, 2003, Cho et al, 2006).

2.4 Examining the Role of HXK2, HXK3, and HKL1 in the Seedling Elongation Response to G6P Treatment

In the interest of wholly understanding the importance of HXK1 in seedling development, we found it important to open a line of investigation into testing the role of other members of its family in hypocotyl elongation; we began under the assumption that HXK1 would have the strongest impact on elongation, HXK2 would demonstrate a reduced but similar impact on growth, HXK3 would have minimal impact, and HKL1 would have an opposite effect on growth. These assumptions were based on previously acquired data in Karve et al 2008 and 2012, discussed in detail in the introduction to this chapter. We started by observing the growth profile of etiolated, short-day photoperiod growth, and constant light 4-day-old seedlings (Fig. 2.4).

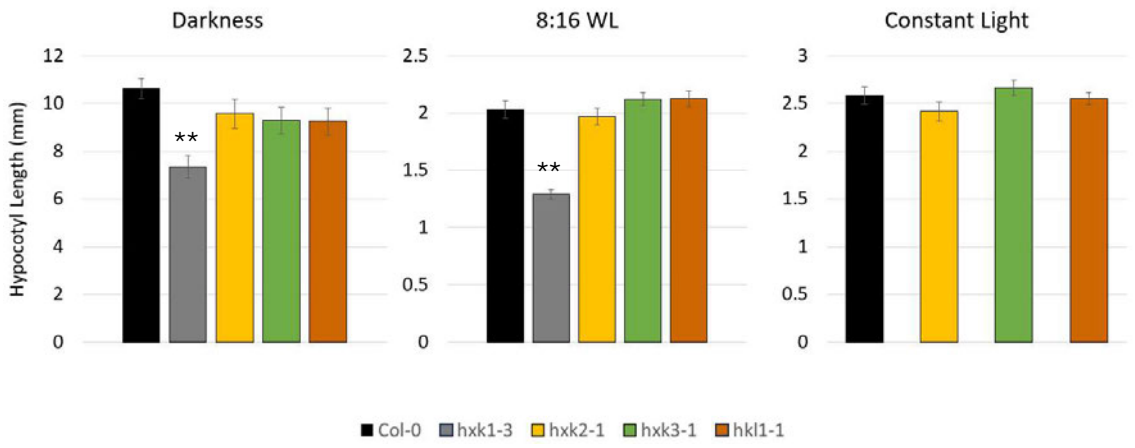

Fig. 2.4 HXK1 Exhibits Significant Control Over Hypocotyl Growth. Various HXK mutant lines grown in darkness (left), 8:16 photoperiodic standard white light (centre), or constant white light ($80m^{-2}s^{-1}$, right) for 4 days on 0.5x MS growth medium. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *hxk#*=HXK#-deficient mutant in Col-0.

Unfortunately, the *hxk1-3* seedlings in constant light treatment were unable to germinate, potentially due to an infection on the plate. In darkness, all mutant lines exhibited a slight reduction in length compared to Wt seedlings, contrary to our expectations based on the literature for *hxk3* and *hkl1*, though these differences are not significant at a threshold of $p \leq 0.01$. However, in short day photoperiod and constant light conditions, we show a dwarfed

hxk1-3 seedling that is not phenocopied by any other mutant line observed in this experiment. We wanted to further explore the importance of G6P in the dark-dependent response of these seedlings and see if these lines responded differently to G6P treatment in short day (8 hours light, 16 hours darkness, abbreviated as SD) photoperiodic conditions as well (Fig. 2.5).

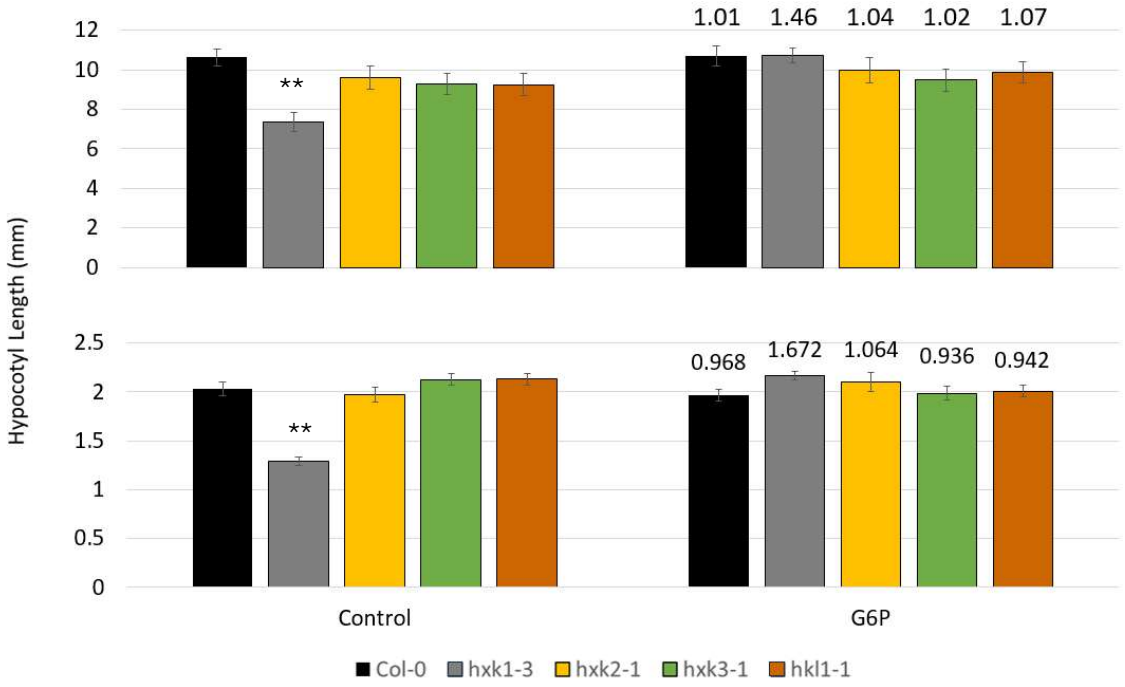

Fig. 2.5 HXK Family Response to G6P Various HXK mutant lines grown in darkness (top) or 8:16 photoperiodic standard white light (bottom), for 4 days on control (left) or 5mM G6P supplemented (right) 0.5x MS growth medium. Numbers above represent fold change from control to G6P for the same genotype. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01. Col-0=Columbia (wild-type) *hxk#*=HXK#-deficient mutant in Col-0.

As shown throughout this chapter, Wt shows minimal response to G6P treatment, only a slight reduction in length that is non-significant in photoperiodic conditions. Similarly reproduced results for *hxk1-3* demonstrate the efficacy of the technique, with its determined elongation response to 5mM G6P being maintained. In darkness, *hxk* mutants other than *hxk1-3* showed very little response to G6P treatment, and though this response is slightly higher than Wt in terms of fold change (FC) it is not a significant difference from their control length. Curiously, in SD conditions *hxk3* and *hkl1* do show a qualitatively different response than *hxk1* and *hxk2* as they shrink with supplemental G6P, though again this difference is non-significant.

2.5 HXK Inhibitor Application Mimics *gin2-1* Mutant in Wild-Type Plants

To further study the nature of the *hxk1-3* and *gin2-1* short hypocotyl, we investigated the use of different HXK inhibitor molecules observed in the literature. According to Hofmann and Roitsch (2000), the inhibitors glucosamine, N-acetyl-D-glucosamine, and mannoheptulose significantly affected the ability of HXK to phosphorylate sugars in vivo, through preferential binding to the HXK glucose-binding domain to prevent phosphorylation of glucose. Initially, we tried to apply N-acetyl-D-glucosamine to growing seedlings but found that at concentrations high enough to show a response in the literature (100mM), no significant changes in hypocotyl length were observed (Fig. 2.6a). In contrast, when mannoheptose, an isomer of mannoheptulose, was used, we were able to significantly reduce the length of Wt hypocotyls in a way that resembled the *gin2-1* and *hxk1-3* mutant phenotypes at applications of 15mM (Fig. 2.6b).

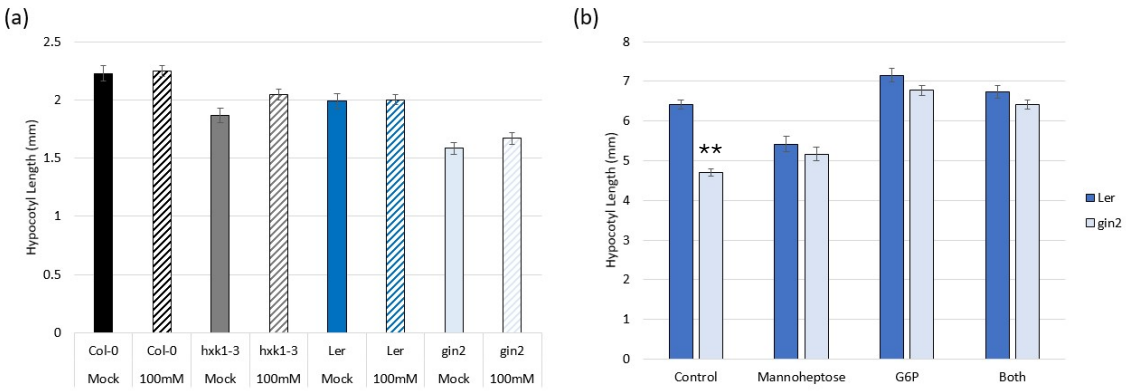

Fig. 2.6 HXK Inhibitor Treatment Phenocopies *hxk1-3/gin2* in Wt. (a) Wt and mutant seedlings grown for 4 days on either control (solid) or 100mM N-acetyl-D-glucosamine (striped) medium in 8:16 photoperiodic standard white light. (b) Etiolated Wt and *gin2-1* seedlings grown on 0.5x MS growth medium for 4 days on control, 15mM mannoheptose inhibitor, 5mM G6P, or growth medium supplemented with both substrates. 15mM mannoheptose was quantified in an earlier test curve not shown here as the minimum required dosage to achieve inhibition of growth when controlled for germination. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant in Ler. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0

Furthermore, the application of G6P in quantities sufficient to restore the mutant hypocotyls back to Wt levels also rescued the mannoheptose-inhibited seedlings. This further supports our previous findings, demonstrating the importance of G6P perception in a normal growth

phenotype and demonstrating the independence of this phenomenon from an unintended effect of mutation. Unfortunately, owing to supplier issues, we were unable to obtain any more mannoheptose for our experiments and were unable to follow this work with it. While looking for alternative inhibitor molecules, we discovered in the literature a potent HXK inhibitor in 2-deoxy-2-glucose (2-DG), which has been demonstrated to inhibit HXK activity in cell cultures (Klein and Stitt, 1998), and can be effectively taken up by seedlings (Jang et al, 1994; Pego et al., 1999). However, this molecule also arrests germination; therefore, we tested whether we could reproduce the inhibitory effects of mannoheptose without lethality at extremely low doses (Fig. 2.7a)

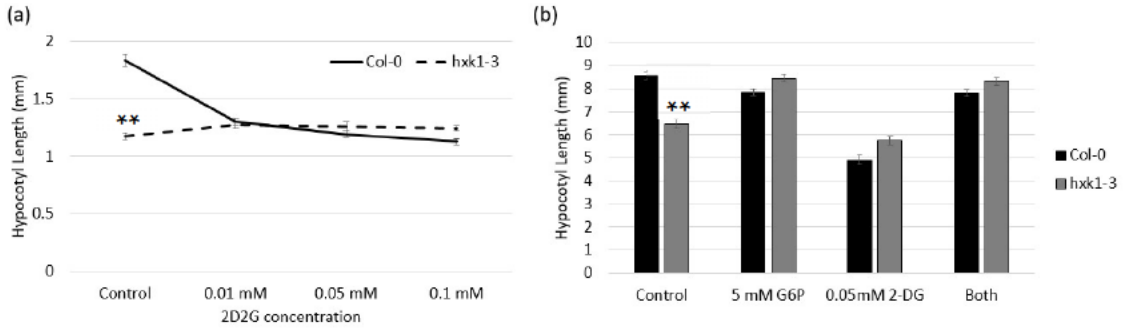

Fig. 2.7 2-Deoxy-D-Glucose HXK Inhibitor Treatment Works *In Vivo*. (a) Dose-response curve of both Wt (solid line) and *hxk1-3* (dashed line) grown in increasing levels of 2-DG for 4 days in standard photoperiodic white light. (b) 4-day old etiolated Wt (black) and *hxk1-3* (grey) seedlings grown on 0.5x MS control, 5mM G6P, 0.05mM 2-DG, or medium supplemented with both substrates. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0

At extremely low dosage, 2-DG supplementation is effective at reducing Wt hypocotyl lengths to *hxk1-3* levels, while minimally affecting mutant seedlings. We found that any dose of 0.5mM or higher was always embryo lethal to both mutant and Wt seedlings (data not shown). To further confirm the efficacy of the inhibitor on limiting growth via HXK inhibition, we subsequently attempted a G6P-dependent rescue of the hypocotyl reduction (Fig. 2.7b) and were successful in recapturing the mannoheptose response using this new molecule. From here, we were able to ask further questions of the effectiveness of HXK1 mediated signalling by applying 2-DG to seedlings expressing the *hxk1*^{S177A} allele. The key information to note in this section is that HXK inhibitor molecules induce a *hxk1* mutant response to various stimuli, providing a useful tool to study the interactions between HXK and other molecules without the need for an exploratory genetic cross, this allows for more

versatility in analysis that can later be verified using genetic crosses. The crosses necessary to make can be narrowed down with this tool to save time and costly efforts to maintain large stocks of potentially irrelevant lines. However, a limitation to this approach may be unintended effects on other proteins within the cell, thus requiring confirmation via these crossed lines.

2.6 HEXOKINASE-mediated Growth is Dependent on the Enzymatic Function

Cho et al. (2006) described two partner proteins that facilitate HXK1 signaling: REGULATORY PARTICLE 5 B (RPT5B) and V-ATPASE B SUBUNIT 1 (VHA-B1 [VAB1]). In this study, they demonstrated that single mutants for these two genes phenocopy the *gin2-1* short hypocotyl phenotype, which further implicates the signalling complex in regulating hypocotyl growth. However, while *hxk1-3* exhibited short hypocotyls as expected, *vha-b1* and *rpt5b* hypocotyls were indistinguishable from Wt hypocotyls (Fig. 2.8a). However, when supplemented with additional glucose (approximately 0.5% w/v), *vha-B1* and *rpt5b* were short compared to Wt, data that matched the published data (Cho et al, 2006). SNF1-RELATED PROTEIN KINASE REGULATORY SUBUNIT GAMMA 1 (KING1 or KINγ) has also been suggested as a signalling component of HXK1 (van Dingenen et al., 2020), but mutant lines showed no significant change in elongation to Wt seedlings under any conditions. Consequently, it appears that for seedlings HXK1 signaling (as directed through VHA-B1 and RPT5B) is only evident when in the presence of exogenous glucose.

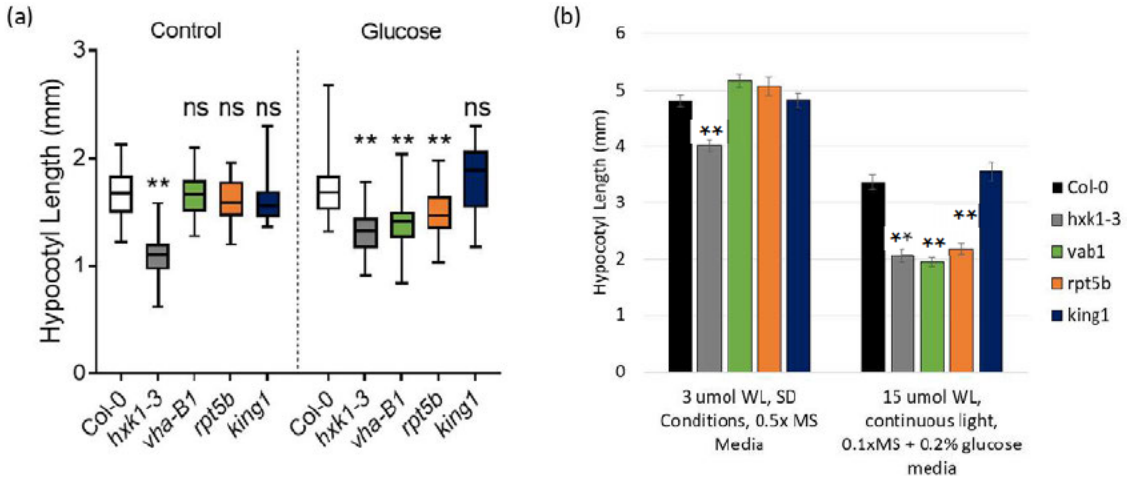

Fig. 2.8 HXK Signaling Component Mutants Regulate Growth on Glucose. (a) Wt and mutant seedlings grown in either control (left) or 28mM (0.5% w/v) glucose supplemented (right) growth medium for 4 days in photoperiodic standard white light. (b) Seedlings grown for 4 days in low ($3m^{-2}s^{-1}$) white light on standard growth medium (left) or in constant moderate ($15m^{-2}s^{-1}$) white light on lighter growth medium supplemented with 0.2% w/v glucose. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *rpt5b* and *vha-b1/vab1* are HXK1 signal complex partners, *king1* is an alternative signal partner.

In analysing HXK1 function many studies have measured the growth of *gin2-1* under high sugar conditions between 2 and 6% weight/volume (w/v); a previous member of the laboratory measured internal glucose levels of plants grown with as little as 0.5% glucose media (g/ml) and reported 6.5-fold internal glucose levels compared to plants grown on un-supplemented media. The conditions described in Cho et al. (2006) were also recreated, and we observed that when we applied their conditions (0.2% glucose, low continuous light), we were able to reproduce their results as well (Fig. 2.8b). Previous work done in our lab has demonstrated that even lower doses of glucose application results in a sizeable increase in internal glucose levels that exceed the physiological by an order of magnitude (Appendix A). Thus, our data implies that HXK1 signaling can mediate hypocotyl growth in seedlings, but only in a high-glucose environment which will be referred to from this point as high glucose (HG) conditions. As a result, we can infer that under physiological conditions the signaling function of HXK1 may not be the dominant operating mode for the protein. These results support our hypothesis on the role of HXK1 in seedlings while explaining the discrepancy between our conclusions with those of others. The important information to note in this section is that we do see an impact of these signaling factors on growth in an HXK-like manner, and thus can replicate published results, but only when exogenous glucose is applied.

2.7 Role of *PFK1* in Seedling Development

With our newfound hypothesis on HXK1 function in the hypocotyl, we proposed examining mutants of genes downstream of HXK1 glycolysis to determine whether we could phenocopy the *gin2-1* short hypocotyl with otherwise impaired glycolysis (Fig, 2.9a). We chose *PHOSPHOFRUCTOKINASE* 1 (*PFK1*) as a target gene, as its mutant is non-lethal and has been described previously in the literature and thus has a confirmed mutant line available for analysis (Perby et al, 2022). We wanted to observe the response of the *pfk1* mutation has on the growth of seedlings in response to G6P and pyruvate (Fig. 2.9b)

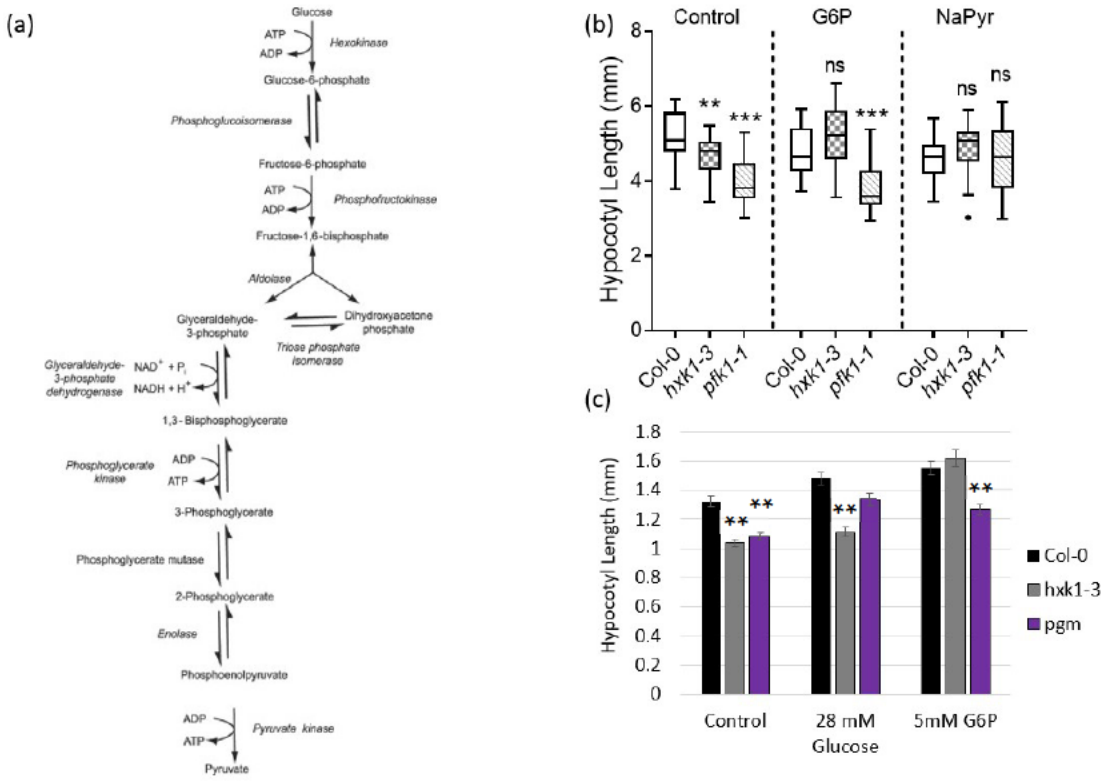

Fig. 2.9 Alternative Metabolic Enzyme Mutants Phenocopy *hxk1-3*. (a) Simplified linear map of glycolysis in *Arabidopsis*. (b) 4-day old Wt (white), *hxk1-3* (checkered), and *pfk1-1* (dashed) seedlings grown in 8:16 low (3 $m^{-2}s^{-1}$) white light on control, 5mM G6P, or 15mM sodium pyruvate supplemented growth medium. (c) 4-day old Wt (black), *hxk1-3* (grey), and *pgm* (purple) seedlings grown in 8:16 standard white light on control, 28mM glucose, or 5mM G6P supplemented 0.5x MS growth medium. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01, ***=pval<0.001. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pfk1-1*=PFK1-deficient mutant in Col-0, *pgm*=PGM-deficient mutant in Col-0

pfk1 mutant seedlings phenocopy *hxk1-3* mutants under physiologically relevant (PR) conditions without any supplemental sugars. As *PFK1* is downstream of G6P production, *pfk1* seedlings did not respond to treatment. However, sodium pyruvate application sufficient to rescue *hxk1-3* short hypocotyls can also rescue the *pfk1* short hypocotyl. Similarly, seedlings lacking *PHOSPHOGLUCOMUTASE* (*PGM*), the enzyme responsible for catalysing G6P to glucose-1-phosphate, phenocopied the *hxk1-3* short hypocotyl and were unresponsive to G6P treatments (Fig. 2.9c) These results support our previous findings and an emerging model of the importance of glycolysis in HXK1-mediated seedling growth. We wanted to further explore the impact of other glycolytically relevant genes in this process, so we looked at the double mutant for *CYTOSOLIC INVERTASE* (*CINV*). *cinv1cinv2* was chosen for this assay due to its importance in glycolysis preceding HXK1 activity (CINV catabolises sucrose into fructose and glucose) and its published role in seedling growth; *cinv1cinv2* mutant seedlings show significantly impaired root growth and an altered response to exogenous sucrose compared to Wt, providing a phenotypic similarity to *gin2-1* (Pignocchi et al, 2020). We then grew these seedlings alongside *hxk1-3* and Wt with and without supplemental glucose and G6P, and measured their hypocotyl growth (Fig. 2.10)

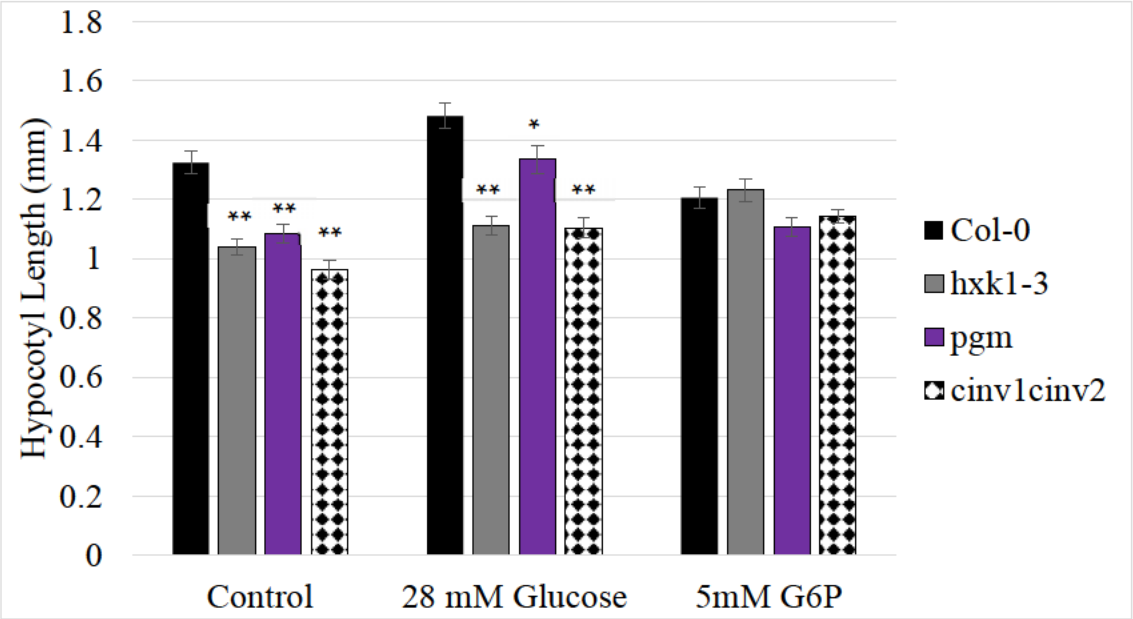

Fig. 2.10 *cinv* Double Mutant Characterization. 4-day old Wt (black), *hxk1-3* (grey), *pgm* (purple), and *cinv1cinv2* (diamond-checkered) seedlings grown in 8:16 standard white light on control, 28mM glucose, or 5mM G6P supplemented 0.5x MS growth medium. Error bars represent standard deviation between samples in three biological replicates. *=pval<0.05, **=pval<0.01. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pgm*=PGM-deficient mutant in Col-0. *cinv1cinv2*=double mutant for two CINV genes in Col-0

In control conditions, we show that *cinv1cinv2* phenocopies the *hxk1-3* short hypocotyl phenotype. This data also shows the established *hxk1-3* response to glucose and G6P as repeatedly shown in this chapter, and Wt responses to these supplements are also as expected. Interestingly, while *cinv1cinv2* seedlings responsive to treatment, they remain significantly shorter than Wt seedlings, unlike *hxk1-3*. This reduced response to G6P is unexpected and suggests that CINV partially regulates growth outside of the G6P pathway, though that it can still induce elongation does demonstrate the importance of maintained glycolysis for seedling growth, consistent with our observations in *pfk1* and *pgm*. This experiment needs repeating, and additional experiments on supplemental sucrose would also prove informative given CINV's role in its catabolism. It is also possible that we might see a stronger response to G6P if we were to test the mutant of the *MITOCHONDRIAL INVERTASE* (*mINV*) gene, as HXK1 is strongly associated with mitochondria and typically localise there (Karve et al, 2008; Martín et al, 2013; Ulfstedt et al, 2018).

2.8 Examining Catalytically Inactive HXK1 Allele Response to Stimuli

We sought to further test our emerging hypothesis by establishing an understanding of the catalytically inactive *HXK1* allele, *hxk1*^{S177A}, as described by Moore et al. (2003). In this study, the S177A point mutation was shown to significantly impair HXK1's ability to phosphorylate glucose in maize protoplasts. Despite its inability to phosphorylate glucose due to a mutation affecting phosphoryl transfer, this mutant allele has been shown to complement the *hxk1-3* short hypocotyl phenotype *in planta*. The authors thus propose that the signaling function of HXK1 is responsible for hypocotyl elongation in low light conditions. Interestingly, when we grew this line under our experimental conditions, we found that *hxk1*^{S177A} complemented the background *hxk1-3* mutation and returned the hypocotyl to Wt length. This data appears to be at odds with our other findings (Fig. 2.11a). However, *hxk1*^{S177A} seedlings exhibited an exaggerated response to glucose when compared to Wt, differing from the *hxk1-3* lagging response (Fig. 2.11b). We see this again when analysed in low light and high light as well, implying that the signaling function of HXK1 is exaggerated in this allele (Fig. 2.11c).

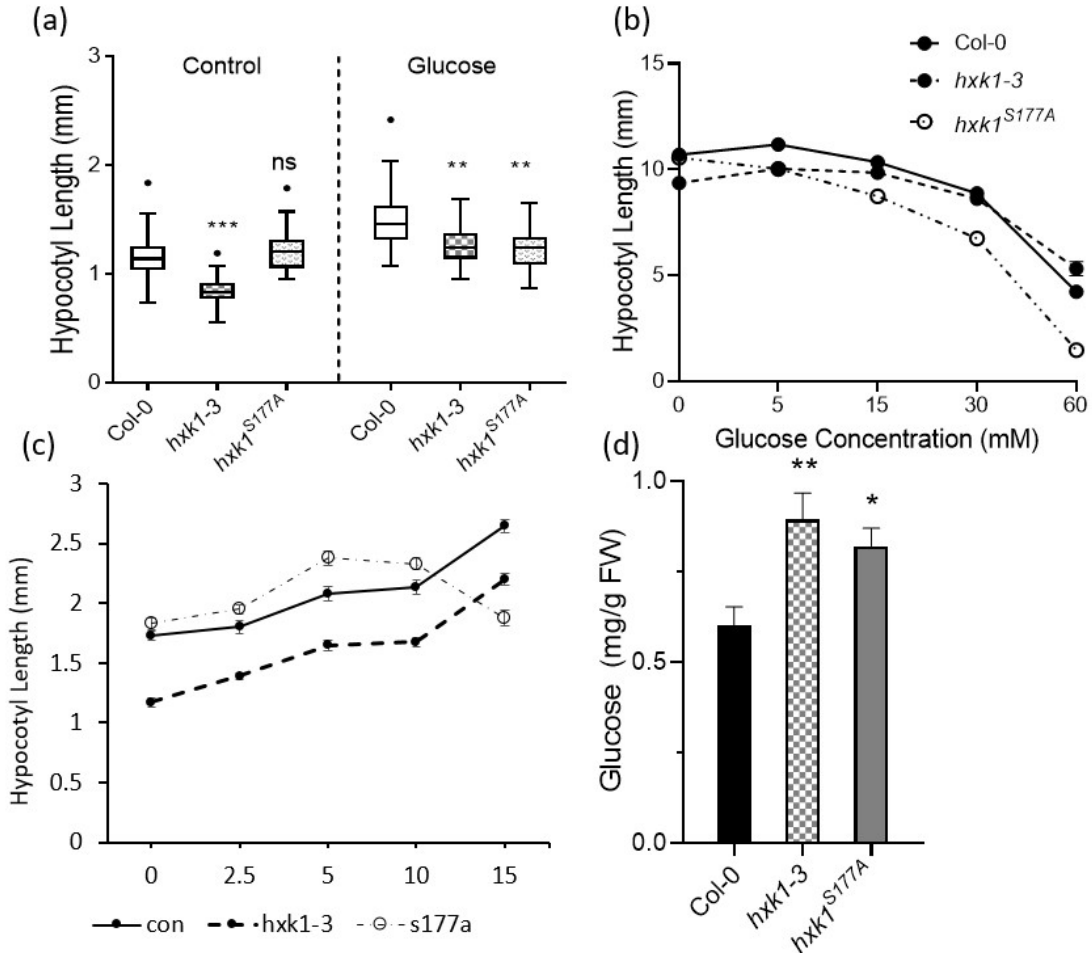

Fig. 2.11 Catalytically Inactive HXK1 Allele Shows Altered Response to Glucose. (a) 4-day old Wt (white), *hxk1-3* (checkered), and *hxk1*^{S177A} (dots) seedlings grown in 8:16 standard white light on either control or 28mM glucose supplemented 0.5x MS growth medium. (b) Glucose dose-response curve of 4-day old etiolated Wt (solid line), *hxk1-3* (dashed line), and *hxk1*^{S177A} (dashed line, white markers) on 0.5x MS medium. (c) Glucose dose-response curve of 4-day old Wt (solid line), *hxk1-3* (dashed line), and *hxk1*^{S177A} (dashed line, white markers) grown in 8:16 standard white light on 0.5x MS medium. (d) Internal glucose levels of Wt (black), *hxk1-3* (checkered), and *hxk1*^{S177A} (grey) seedlings grown in 8:16 standard white light on glucose-free 0.5x MS medium, normalized to fresh weight. Error bars represent standard deviation between samples in three biological replicates. *=pval<0.05, **=pval<0.01, ***=pval<0.001. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *hxk1*^{S177A}*/s177a*= catalytically inactive HXK1 allele expressed in *hxk1-3*

Previous work in the laboratory demonstrated that *gin2-1* seedlings show an elevated level of glucose compared to Wt seedlings (Appendix A, Lincoln et al., 2023). When we quantified the internal sugar levels of *hxk1*^{S177A} seedlings, we found that the background mutation in the *hxk1*^{S177A} allele retained increased levels of glucose (Fig. 6d). In the context of our other experiments, this indicates that the capacity of the mutant allele to rescue the *hxk1* short

hypocotyl is due to an activation of the HXK1 signalling capacity from the elevated levels of sugar. This would also explain previous findings on the importance of the signalling role of HXK1 in controlling hypocotyl elongation, as most, if not all, physiological work has been performed under HG conditions. It is also possible that residual kinase function may be present in the protein, which could then rescue to phenotype, as predicted by Feng et al (2015).

To further test the potential ways in which *hxk1*^{S177A} could complement the *hxk1-3* mutation, we wanted to observe its response to HXK inhibitor 2-DG and G6P; if *hxk1*^{S177A} is able to fully complement the mutation on its ability to signal with the enhanced internal glucose, then the seedlings should have a Wt response to G6P and an insensitivity to 2-DG application (Fig. 2.12).

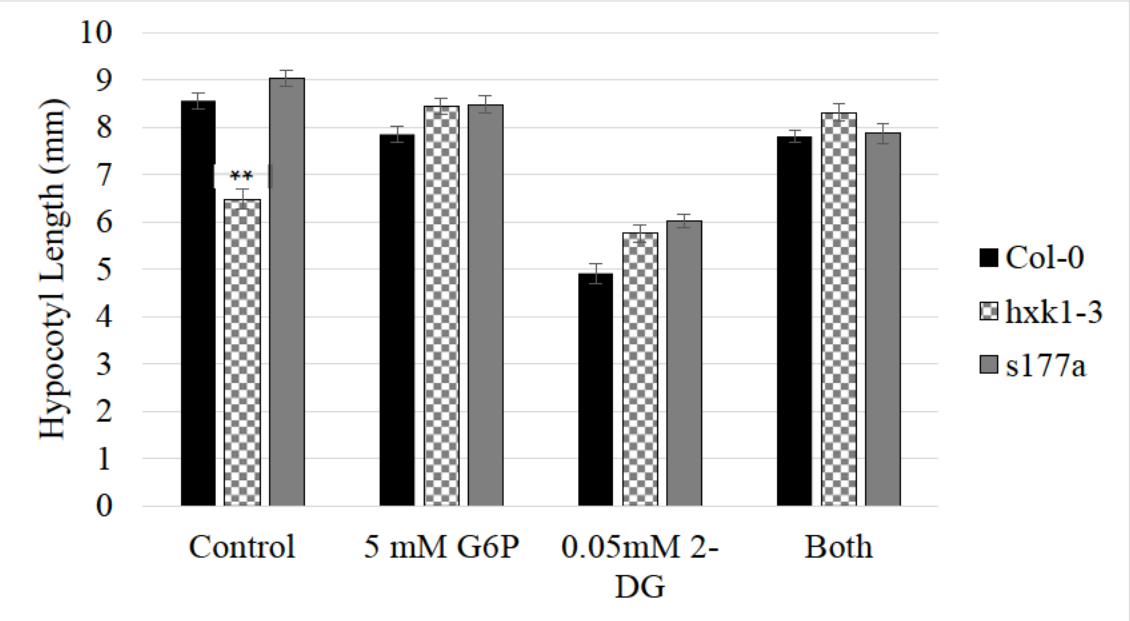

Fig. 2.12 Catalytically Inactive HXK1 Allele Response to 2-DG Inhibitor. 4-day old, etiolated seedlings of Wt (black), *hxk1-3* (checkered), and *hxk1*^{S177A} (grey) grown in control, 5mM G6P, 0.05mM 2-DG, or growth medium supplemented with both substrates, all with 0.5x MS. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.001. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *s177a*= catalytically inactive HXK1 allele expressed in *hxk1-3*

As previously observed, *hxk1*^{S177A} complements the mutation in control conditions, and the Wt and *hxk1-3* responses to treatments are consistent with prior observations. However, while *hxk1*^{S177A} shows a Wt response to G6P, it also exhibits a Wt response to both 2-DG inhibitor

as well as the dual treatment, contrary to our expectations going into this experiment. This experiment suggests that there may in fact be some residual kinase function in this allele.

2.9 Associations with PIF Signaling

Similar to HXK1, light signaling significantly regulates hypocotyl development (Leivar et al., 2008; Park et al., 2012; Park et al., 2018; Lilley et al., 2012). Given the pronounced phenotypic resemblance between etiolated hypocotyls lacking red-light perception and mutants affecting sugar signaling, our focus shifted towards exploring potential co-regulation levels between these two pathways. PHYTOCHROME (PHY) primarily detects red light, directly modulating the activity of PHYTOCHROME INTERACTING FACTORS (PIFs) (Leivar et al., 2008; Park et al., 2012; Park et al., 2018; Lilley et al., 2012). The quadruple *PIF* mutant *pifQ*, a cross between individual PIF mutants *pif1-1*, *pif3-1*, *pif4-2*, and *pif5-3*, demonstrated a qualitatively similar response to increasing fluences of light to *hxk1-3* and *gin2-1* mutants (Fig. 2.13).

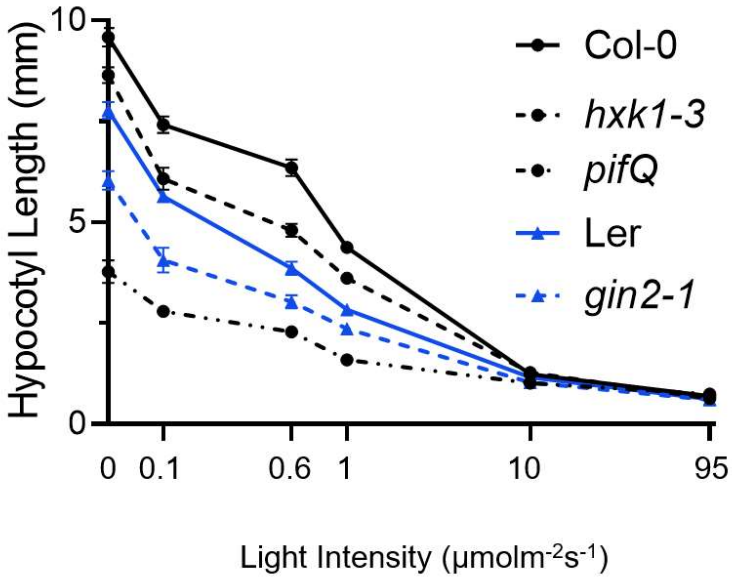

Fig. 2.13 Phenotypic Similarities in Skotomorphogenesis for HXK1 and PIFs. Wt (solid lines) and mutant (dashed lines) seedlings grown in constant light of increasing intensity for 4 days on 0.5x MS medium. Colours assigned to relevant ecotypes; blue to *Landsberg erecta*, black to *Columbia-0.* Error bars represent standard deviation between samples in three biological replicates. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant in Ler. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0

The similar nature of this response coupled with the fact that PHY and PIFs have been implicated in carbon resource partitioning (Yang et al., 2016; de Wit et al., 2018; Krahmer et al., 2021) suggest that there might be cross regulation between the two pathways. We wanted to test the responsiveness of *pifQ* seedlings to exogenously applied G6P to determine if the short hypocotyl of these seedlings could possibly be attributed to changes in *HXK1* expression or even protein activity (Fig. 2.14). We chose to analyse the effects of G6P on the length of individual cells, as we knew from previous work in the lab that the *gin2-1* mutation resulted in significantly smaller epidermal cells across the hypocotyl (Appendix A).

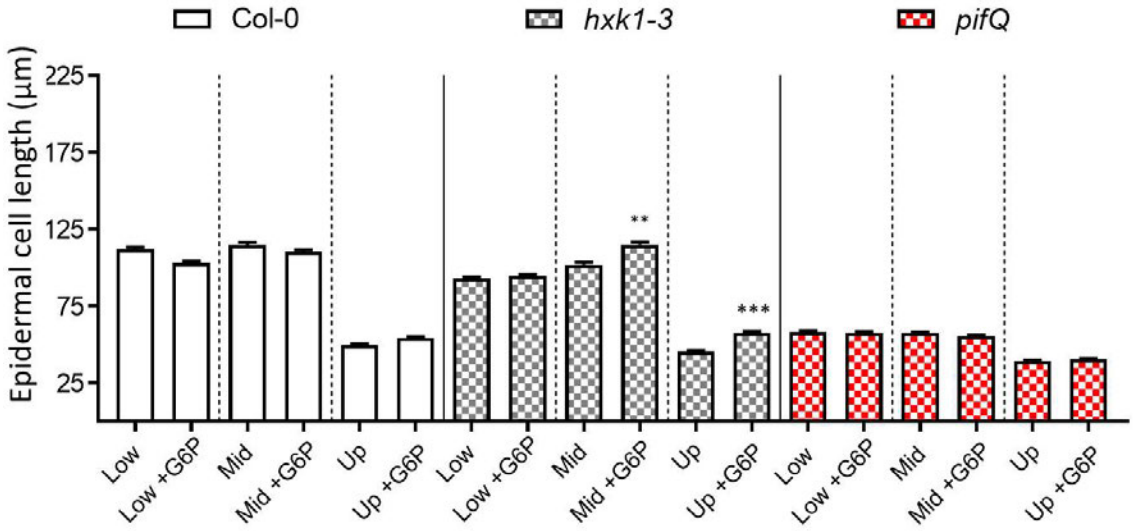

Fig. 2.14 Cell Growth Affected by HXK1 Dependent on G6P While PIFs Insensitive. Epidermal cell size and count of Col-0, *hxk1-3*, and *pifQ* seedlings grown in low fluences (3 µmol $m^{-2}s^{-1}$) 8:16 white light on 0.5x MS medium without (left) or with 5mM G6P (right), cells clustered and measured in average based on proximity to the shoot apical meristem (Up), radicle (Low), or neither (Mid). Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01, ***=pval<0.001. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0

However, *pifQ* seedlings were unresponsive to the quantities of G6P that rescued *hxk1-3* seedlings, showing no significant change at the cellular level at all sampled cell types. To further explore the effects that G6P may or may not have on *pifQ* seedlings, we wanted to take a closer look at the expression of genes known to be misregulated in etiolated *pifQ* seedlings as described in the literature (Zhang et al, 2013). The extent of this analysis will be described in the following chapter of this thesis. Further study of the interaction between

these pathways indicates that there is more nuance to the interplay between light signalling and HXK1-mediated growth, which will be explored in chapter 4 of this thesis.

2.10 Discussion

The investigation of signal convergence and growth in the *Arabidopsis* model system, particularly focusing on the seedling hypocotyl, has been addressed through various studies (Singh et al, 2017; Ivakov et al, 2017; Chen et al, 2008). The growth of the hypocotyl is a significant indicator of these signals' effects. Post-germinative expansion of cells necessitates well-coordinated signals and relies on the utilization of seed reserves alongside signaling responses (Short, 1999; Penfield et al, 2004; Stewart et al, 2011). The present research underscores the role of HXK1 in this process by means of glycolytic activity.

HXK1 has been acknowledged to possess two distinct functions: firstly, as a glycolytic enzyme that facilitates the conversion of glucose to G6P, and secondly, as a sugar-sensing molecule localized in the nucleus (Moore et al, 2003; Cho et al, 2006). While its involvement in the development of seedling hypocotyls has predominantly been attributed to its nuclear signaling role, this study reveals that when external glucose supply is absent, HXK1's glycolytic activity becomes crucial for supporting hypocotyl growth. This becomes particularly evident under conditions of limited light, whether due to shorter diurnal light periods or reduced light intensity in both continuous and diurnal light cycles. Unlike glucose, G6P exhibits a selective ability to restore the shortened hypocotyl of the *gin2-1/hxk1-3* mutants to a normal length. These findings highlight the significance of HXK1’s production of G6P, and thus indirectly suggests an importance in its capacity to break down carbon reserves derived from seeds, providing the necessary fuel for hypocotyl expansion when light availability is restricted.

The conversion of triacylglycerols and storage proteins into sucrose contributes to glycolysis, thus facilitating the generation of energy, carbon skeletons, and metabolites essential for cell growth (Penfield et al, 2004; Graham, 2008). Furthermore, when germinating in darkness/low light conditions, seed reserves act as fuel for hypocotyl growth until light is perceived, which then inhibits hypocotyl extension while promoting cotyledon opening and greening which are required to switch to photoautotrophic growth (Holm and Deng, 1999; Leivar and Quail, 2011). In our experiments, we observe HXK1-G6P mediated expansion of epidermal cells in seedling hypocotyls grown in light-limiting conditions, demonstrating an important role for HXK1 in driving skotomorphogenic growth.

Our results with the *HXK* family genes show little effect of any of these genes on the hypocotyl elongation response. These results are largely consistent with what little is known about them in the literature, with one discrepancy. Karve and Moore (2009) do show that *hkl1* hypocotyls are longer than Wt seedlings, but this difference is quite subtle and their growth conditions are different to ours in terms of nutrient quantity in the growth medium (1/5x in the study, 0.5x in ours), light levels (15 μmol m−2 s−1 constant light in the study, 80 μmol m−2 s−1 in ours), and temperature (22 °C in the study, 20 °C in ours). It is possible that under these conditions HKL is more impactful in seedling growth, but for the purposes of this study in comparing its role to that of HXK1, we see no strong role for HKL in growth in conditions that HXK1 does. Likewise, HXK2 and HXK3 seem to have little impact on hypocotyl elongation in these conditions. This could be due to a variety of factors: lower "importance" during this life stage of these genes giving less impact on growth, gene redundancy with HXK1 allowing it to replace them where they are missing. Given more time, we would like to finalize our genetic crosses between mutant lines *hxk1*x*hxk2* and *hxk1* x *hxk3* to see if a cumulative effect of the loss of function to both genes could have a wider impact, which would give credence to the latter theory. In a later chapter, we will analyse the expression of these *HXK* family genes in a different context; the qPCR data seems to indicate a higher abundancy of *HXK2*/*PP2A* compared to *HXK1*/*PP2A* in these young seedlings, which seems to imply that *HXK2* may be performing some unknown function at this time in seedlings unrelated to hypocotyl growth (Fig. 5.2). There is much work that needs to be done to fully understand the intricacies of how this gene family operates at the seedling level, and it begins with the establishment of several double mutant lines and a triple mutant line between the three HXK genes.

Previous research has established that VHA-B1 and RPT5B, in conjunction with HXK1, constitute a nuclear complex that directly connects glucose perception to nuclear signaling and the growth of hypocotyls (Cho et al, 2006). Consistent with this concept, we observe a defect in hypocotyl growth in mutants lacking VHA-BA and RPT5B, but this occurs only when external glucose is introduced. In contrast, the shortened hypocotyl phenotype observed in the *gin2-1/hxk1-3* mutant is evident irrespective of glucose availability. Cumulatively, the data suggests that post-germinative hypocotyl elongation is regulated through both the glycolytic and sensor-signaling pathways of HXK1. However, before the establishment of photoautotrophic growth, the dominance of the glycolytic pathway is evident, as VHA-B1

and RPT5B do not appear to have a role in skotomorphogenesis. This could be further tested with a double mutant line to establish distinction.

In our study evidence has been presented for HXK1's glycolytic regulation of hypocotyl growth. However, the *hxk1*^{S177A} mutant, which lacks catalytic activity, does not exhibit the anticipated short hypocotyl phenotype under low light conditions that our data would suggest, aligning more with previous studies (Moore et al, 2003). This led us to hypothesize that similar to *gin2-1* and *hxk1-3,* the loss of enzymatic activity might result in elevated internal glucose levels. This elevation could trigger glucose signaling and subsequent hypocotyl elongation, effectively concealing any growth impairment stemming from the absence of enzymatic function (Kim et al, 2013). Our analysis, which unveiled heightened glucose levels in the *hxk1*^{S177A} mutant alongside an altered glucose response curve, lends support to this hypothesis, providing a plausible explanation for the *hxk1*^{S177A} phenotype. It is also possible that a greater pool of available HXK1 would be available for signaling purposes in this case, prematurely activating the signaling role. If this were the case, we would expect the catalytically inactive allele to have an altered response to glucose as a result of this induced signal.

In support of this notion, our results demonstrate an enhanced glucose response in the *hxk1*^{S177A} line; it is therefore likely that rather than complementing the *hxk1-3* mutation, the response to the elevated glucose and induced signal from the large pool of available HXK1 was effective in masking the effects of *hxk1-3*. Our experiment with HXK1 inhibitor application to the *hxk1*^{S177A} line demonstrates that it significantly responds to inhibitor treatment; our previous work would suggest that the activated signaling from previously described factors should be unperturbed by an inhibitor molecule in the system. This could be explained by the way in which the inhibitor molecule works; as mentioned previously, 2-DG competitively binds to the glucose site in HXK, preventing the catabolism of sugar through HXK (Klein and Stitt, 1998). It is therefore possible that the binding of sugar is required for the initiation of HXK1 signaling, and thus 2-DG is equally effective at limiting both functions of HXK1. Nonetheless, G6P does successfully rescue the impaired growth in the *hxk1*^{S177A} seedlings, which does support the importance of HXK1 enzymatic activity in seedling growth even when the signaling role is the only active method.

This could be further explored in several ways. The first experiment we considered would be to examine the glucose response of the double mutant for signaling components *VHA-B1* and

RPT5B, which should result in an environment with no HXK1 signaling but maintained catabolic activity of the native protein. If our hypothesis is true, we would expect a Wt phenotype in control and an altered response to increasing glucose levels (as HXK1 signaling would be activated in Wt). Another experiment to consider is expressing a foreign glucokinase, which has been successfully done in *Arabidopsis* using the glucokinase of a charophyte algae (Ulfstedt et al, 2018). There are glucokinases which are catalytically active but have no signaling role, and thus would not contribute to a nuclear signal when crossed into the $hxk1^{S177A}$ line. We could then quantify if the internal levels of glucose were reduced back to a Wt state, and then test the $hxk1^{S177A}$ response to increasing doses of glucose; if our hypothesis on the method $hxk1^{S177A}$ promotes growth is true, we would expect a Wt phenotype as the pool of available glucose would be smaller, and if our assumption is inaccurate, we would expect an altered response to glucose.

Further backing for the significance of glycolysis in seedling skotomorphogenesis is derived from growth analysis demonstrating that the glycolytic enzyme mutant *pfk1-1* replicates the *hxk1-3* phenotype; moreover, both mutants respond positively to external pyruvate application. This further establishes the importance of HXK1 in the mobilisation of seedling reserves in order to fuel growth at the onset of seedling development, a very important role at this stage of development that affects growth at further life stages (van Dingenen et al, 2018).

The phenotypic similarities between *gin2-1* and *pifQ* in skotomorphogenesis and increasing light led us to believe that there might be significant overlap between PIF and HXK1 in regulating key growth factors at this stage. PIFs and sucrose signaling have been repeatedly shown to be tied together in significant ways; PIF5 has been shown to mediate the effects of sucrose by permitting growth dependent on exogenous sucrose via its expression or lack thereof (Stewart et al, 2011). Likewise, HXK1 and PIF4 have been recently linked together in the control of hypocotyl elongation via guard cell-based interactions, where PIF4 is shown to be epistatic to HXK1 in this process (Kelly et al, 2022). This would lead us to believe that a *pifQ* mutant seedling would respond to G6P in a similar way to the *gin2-1* mutant, but our results demonstrate otherwise. Prior research has implicated PIFs in sucrose-dependent elongation of hypocotyls and the expression of auxin-related genes, with PIF5 playing a prominent role (Stewart et al, 2011; Lilley et al, 2012). Consequently, there was interest in determining whether PIFs have a broader function in integrating light and HXK1 signals to regulate growth. The analysis indicates that the *pifQ* mutant, characterized by short hypocotyls, displays complete insensitivity to G6P, suggesting that G6P is not a limiting

factor for growth in *pifQ* mutants. However, given that *pifQ* represents only four out of the eight PIF proteins in *Arabidopsis*, it remains plausible that other PIF-mediated growth deficiencies operate in a manner responsive to HXK1-G6P. At this life stage, PIF growth promotion appears to work through a distinct pathway to that of HXK1-mediated growth, as PIF mutants are unresponsive to treatments that alleviate HXK mutant phenotypes. In the following chapter, we will further explore alternative areas of overlap between HXK1 and PIF by examining similarities and differences in the transcriptomes of etiolated mutant seedlings.

2.10 Conclusion

In this chapter, we have established the importance of HXK1's enzymatic role in the promotion of seedling growth through several different approaches. By highlighting the response of *hxk1-3* and *gin2-1* mutant seedlings to G6P supplementation, we demonstrate that it is the establishment of G6P synthesis that is critical for hypocotyl development, and we can further establish this principle by utilising several different glycolytic inhibitor molecules to phenocopy the mutant phenotype while also rescuing with G6P supplementation. This shows that the effectiveness of G6P supplementation is not due to unintended consequences of the insertion event in the tDNA *hxk1-3* line or the mutagenesis that produced the *gin2-1* mutant line.

By examining the importance of downstream components in G6P-responsive pathways, we can determine that it is the completion of the cycle that is impactful in seedling growth. That we can replicate published results in key HXK papers indicates that our experimental methods and growth conditions are sound, and that conclusions reached in previous studies have backing in the right conditions to elicit HXK1 signaling. Our exploration of PIF signaling revealed intriguing connections between the red-light signaling pathway and HXK1-mediated growth. The *pifQ* mutant's response paralleled the phenotypic characteristics of *gin2-1* under varying light conditions, hinting at potential crosstalk between these pathways. However, the distinct responses of *pifQ* to glucose and G6P supplementation, coupled with the insensitivity to G6P observed in *pifQ* cells, suggest a complex interplay that warrants further investigation. One possible conclusion to draw from this would be that HXK1 signaling drives growth once photosynthesis is activated and internal sugar levels are elevated, and that in the seedling it is enzymatic activity that promotes growth from catabolising seed reserves.

Chapter 3: *HXK1* Mutant Transcriptomics Analysis

3.1 Introduction

HXK1 misregulation has been repeatedly demonstrated to disrupt glucose-based regulation of several genes, most notably the photosynthetic genes *CAB2* and *CAA* (Moore et al, 2003; Cho et al, 2006). It is important to note that these experiments in MS medium on seedling hypocotyls were invariably performed in conditions of artificially elevated glucose; in the previous chapter, our work highlights the importance of studying the role *HXK1* plays in development under more physiologically relevant conditions. In the course of our investigations into the role that *HXK1* plays in seedling development, a previous member of the Halliday lab performed an mRNA-sequence of 4-day-old etiolated *gin2-1* seedlings in order to further study the gene's impact on the transcriptome in physiological conditions, that is on medium with no additional glucose, and cross referenced this sequence to Wt seeds to generate a sizable list of misregulated genes. The full dataset can be found in appendix A as a part of a manuscript for submission.

Sugar-mediated genetic regulation is a well-established subset of research; the earliest genome-wide profiling of *Arabidopsis* seedlings with sucrose as a supplement was performed in 2005, and demonstrated how sucrose mitigated the effects of anoxia on auxin response genes, laying out an important sequence of studies to follow exploring the interactions between sucrose, starvation, and auxin (Loreti et al, 2005; Gonzali et al, 2006; Lilley et al, 2012, Barbier et al, 2015). Indeed, it is the implications of sucrose signaling acting on other pathways that drives a significant amount of research into understanding the underlying mechanisms behind sugar signaling. Recent studies have shown that *SnRK1*-mediated sucrose signaling is critical to development in plant seedlings through interactions with the circadian clock (Simon et al, 2018; Román et al, 2021; Avidan et al, 2023). Thus, it becomes all the more important to understand the ways that sugar-mediated signals interact with other signaling pathways in order to broaden our perspective on sugar-mediated growth. A great deal of information is currently being gathered on the importance of SnRK1 mediation of stress response and development; a recent landmark publication demonstrated the importance of SnRK1 activity in plants is pronounced to the point of alterations in a highly conserved energy sensor complex to better suite the plasticity of plants via nuclear translocation (Ramon et al, 2019). However, a great deal of information remains unclear on the importance of

HXK1 and how its regulation network may feed into and respond to other signals. Recently, some studies have begun to explore various crossings between HXK and other signaling pathways. A whole genome transcriptional profile revealed that brassinosteroid (BR) and glucose signals intersect at a significant capacity; 72% of all BR-regulated genes were also glucose regulated (a majority of these overlaps being in the same direction), including genes involved in BR metabolism (Gupta et al, 2015). Cytokinin signaling has also been demonstrated to have significant overlap with glucose in root development of *Arabidopsis*, presumed to be mediated through auxin as a nodal point downstream of each pathway (Kushwah and Laxmi, 2017). Other hormone signals, such as ABA and auxin, have also been demonstrated to interact with glucose; auxin and glucose both have strong effects on root development, and glucose has been shown to significantly affect the expression of key genes involved with auxin transport and auxin-induced cell division (Mishra et al, 2009; Wang and Ruan, 2013). Glucose and ABA both exhibit significant control over seed germination as well, with glucose-delayed seed germination being dependent upon ABA (Rolland et al, 2006). Very recently, a study in strawberry plants demonstrated that HXK1 kinase activity is essential to bridging drought stress and sugar metabolism (Wu et al, 2023). These studies start to examine the ways in which glucose via HXK1 impacts and interacts with other signaling pathways, but this work is only just beginning.

In this chapter, I will describe my analysis of this dataset, cross-reference it to published sequencing data to underly patterns with other gene networks, and present notable conclusions we can derive from this dataset within the context of what we now know about HXK1 activity in the seedling.

3.2 General Trends in the mRNA seq Data

In an RNA-seq experiment on 4-day old etiolated *gin2-1* and Wt seedlings, we identified 2344 genes that showed significant misregulation (logFC > 0.58, p < 0.01, and FDR < 0.05) in *gin2-1* compared to Ler (Fig. 3.1). Out of these, 1156 genes were downregulated, and 1188 genes were upregulated when compared to Wt. Analysis of the downregulated gene category revealed associations with lipid storage, ribosome biogenesis, and cell proliferation, indicating energy-consuming processes. Conversely, the upregulated gene category exhibited a pronounced enrichment of genes involved in cellular responses to starvation, cellular respiration, ATP biosynthesis, amino acid catabolism, and photosynthesis. These findings suggest a starvation-type response and a diminished capacity for sugar catabolism.

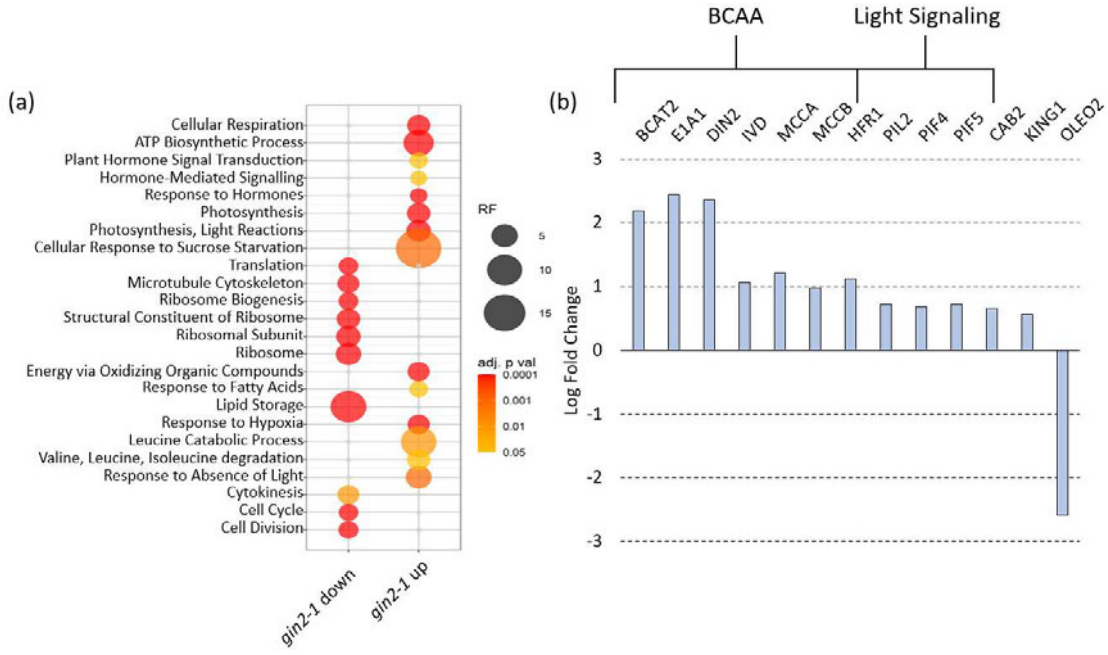

Fig. 3.1 RNA-seq Results Show Significant Effects on Metabolism and Starvation Responses in *gin2-1*. (a) Bubble plot for biological process gene ontology (GO) terms enriched in the *gin2-1* RNA-seq experiment for the manuscript seen in Appendix A (Lincoln et al, 2023). (b) Plotted logFC from *gin2-1* to Wt for several genes of interest. 4-day old *gin2-1* and Wt seedlings were grown in darkness on 0.5x MS medium and were sampled at ZT4. *gin2-1*=HXK1-deficient mutant in Ler. BCAA=branched-chain amino acid catabolism genes

Notably, strongly upregulated genes in *gin2-1* included enzymes related to the catabolism of branched-chain amino acids (BCAA) genes: *BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2 (BCAT2), DARK INDUCIBLE 2 (DIN2), AT1G21400 (E1A1), ISOVALERYL-COA-DEHYDROGENASE (IVD), AT1G03090 (MCCA),* and *3-METHYLCROTONYL-COA CARBOXYLASE (MCCB)*. *BCAA* genes are typically suppressed

in carbon-rich conditions but induced by stress or starvation, providing alternative substrates for respiration (Binder, 2010). This suggests a role for *HXK1* in antagonizing the glucose response. Additionally, our results indicate that, similar to hypocotyl growth, G6P application has minimal impact on BCAA gene expression in Wt but effectively restores expression to Wt levels in *gin2-1* (Appendix A). This observation implies that BCAA gene expression is regulated by the HXK1-G6P pathway. Furthermore, other genes of interest we note in this transcriptome are light signaling genes, such as *LONG HYPOCOTYL IN FAR-RED* (*HFR1), PHYTOCHROME INTERACTING FACTOR 3-LIKE 2 (PIL2), PIF4,* and *PIF5*, canonical sugar-responsive genes like *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2)*, the HXK1 associated signal partner gene *SNF1-RELATED PROTEIN KINASE REGULATORY SUBUNIT GAMMA 1* (*KING1)*, and lipid storage genes like *OLEOSIN 2* (*OLEO2)*. The significance of these genes will become apparent further in this chapter as we cross-reference their expression in other mutant lines.

3.3 Effect of *gin2-1* Mutation on Plastome

A section of particular interest we noted in the analysis of this transcriptome is that a significant majority of plastomic genes are misregulated in *gin2-1*: 82.8% of the chloroplast genome and 23.5% of the mitochondrial genome (Fig 3.2a). Within the mitochondria genome, there is enrichment in genes that oversee metabolic proton and electron transport misregulated in our dataset. Within the chloroplast, we find an abundance of genes except for 23 specifically related to vesicle formation and chromatin remodelling. To explore the significance of this misregulation, we wanted to quantify the effect of the *gin2-1* mutation on the greening of seedlings being transferred from darkness into the light, either at low or moderately high fluences (Fig. 3.2b).

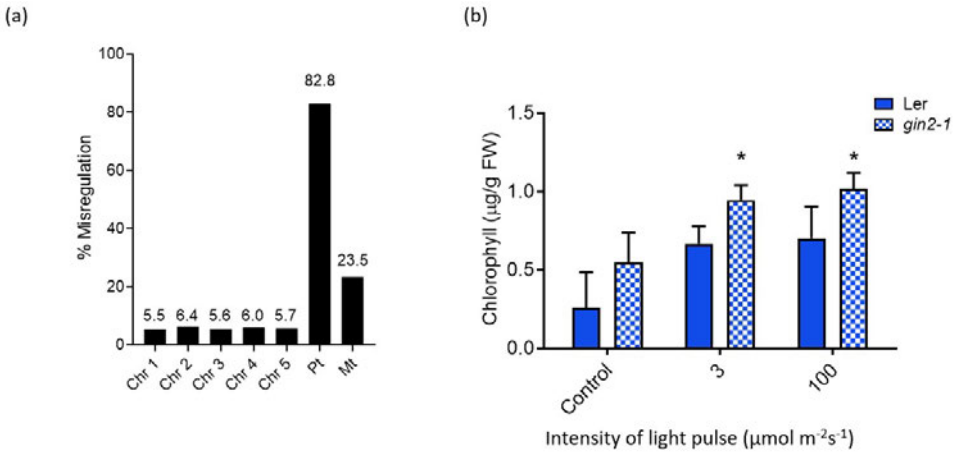

Fig. 3.2 *gin2-1* Has Significant Effect on the Plastome Which Influences Chlorophyll Abundance. (a) Percentage of genes from each chromosome misregulated in *gin2-1* compared to Wt. Pt = chloroplast genome, Mt = mitochondrial genome (b) Chlorophyll/protochlorophyllide content of *gin2-1 (*checkered*)* and Wt (solid) seedlings. Seedlings grown in darkness (left) or transferred to either 3 (middle) or 100 (right) µmol $m^{-2}s^{-1}$ white light for 1 hour. Error bars represent standard deviation between samples in two biological replicates. *=0.01<pval<0.05. Ler=*Landsberg erecta* (wild-type) *gin2-1*=HXK1-deficient mutant in Ler.

In this experiment, we note that *gin2-1* showed non-significantly higher levels of protochlorophyllide in etiolated seedlings compared to Wt, but when transferred into both very low and higher constant light treatments showed significantly increased abundance of chlorophyll, suggesting that the increased transcription of the chloroplast genome in these seedlings is impactful in their de-etiolation response, and resolves to increase the greening potential of these seedlings when compared to Wt. While the difference between *gin2-1* and Wt is statistically insignificant in the control, the biological significance of this phenomenon is apparent from the consistent effects of the mutation on the plastome.

3.4 Exploration of SnRK1 HXK1 Intersection

Due to the enrichment of starvation-associated genes in the RNA-seq analysis, we wanted to explore the potential cross-regulatory processes between HXK1 and the master plant sugar response hub SNF-RELATED KINASE 1 (SnRK1).

In an RNA-seq experiment on 4 day-old etiolated seedlings with a SrRK1 subunit double mutation for alpha 1 and alpha 2 *(SnRK1a1a2)* harvested at the end of the night, it was discovered that there were 4508 and 3510 (8018 total) genes were differentially up- and downregulated with a $\log_2FC>0.5$ or a $Log_2FC<-0.5$; with no cutoff, they observed 6554 upregulated and 5725 downregulated genes, totalling 12304 genes (Henninger et al, 2021). In the upregulated cluster, they observed strong enrichment in genes associated with metabolism and hormonal responses (GO:006749, 0009751), and in the downregulated genes enrichment in photosynthetic genes and light-responsive genes (GO:0015979, 0009416) (Fig. 3.3).

Fig. 3.3 *SnRK1a1a2* transcriptomic analysis. Gene ontology terms enriched in the *SnRK1a1a2* dataset from Henninger et al (2021), either (a) upregulated (or (b) downregulated

Some particular genes of interest we noted in this transcriptome were the BCAA genes previously described (GO:0009063), which were ubiquitously repressed in the mutant, and

other genes relevant to HXK1 signaling such as *KING1* (described as a potential partner protein for HXK1 signaling in the cytosol [van Dingenen et al, 2019]) and *VHA*-B1 (shown to bind to HXK1 and facilitate nuclear signaling [Cho et al, 2006]).

HXK1's activity as an enzyme places it upstream of SnRK1 in metabolism, so associations between a loss of function allele for both genes make logical sense if they operate within the same signaling pathways; of the 5854 significantly misregulated genes in *gin2-1* with a logFC>0.4 or <-0.4, 2847 (48.63%) are also identified as being misregulated by SnRK1 in etiolated seedlings with the same logFC parameters. If HXK1 and SnRK1 operate within the same pathway, we would expect to see a majority of shared misregulated genes having opposing regulation as HXK1-G6P activity is directly inhibitory to SnRK1 kinase activity (Fichtner et al, 2020). 291 genes are downregulated in both, 470 are mutually upregulated, 1004 are upregulated in *SnRK1a1a2* but down in *gin2-1*, and 1073 are downregulated in *SnRK1a1a2* but up in *gin2-1*, consistent with our expectation (Fig. 3.4).

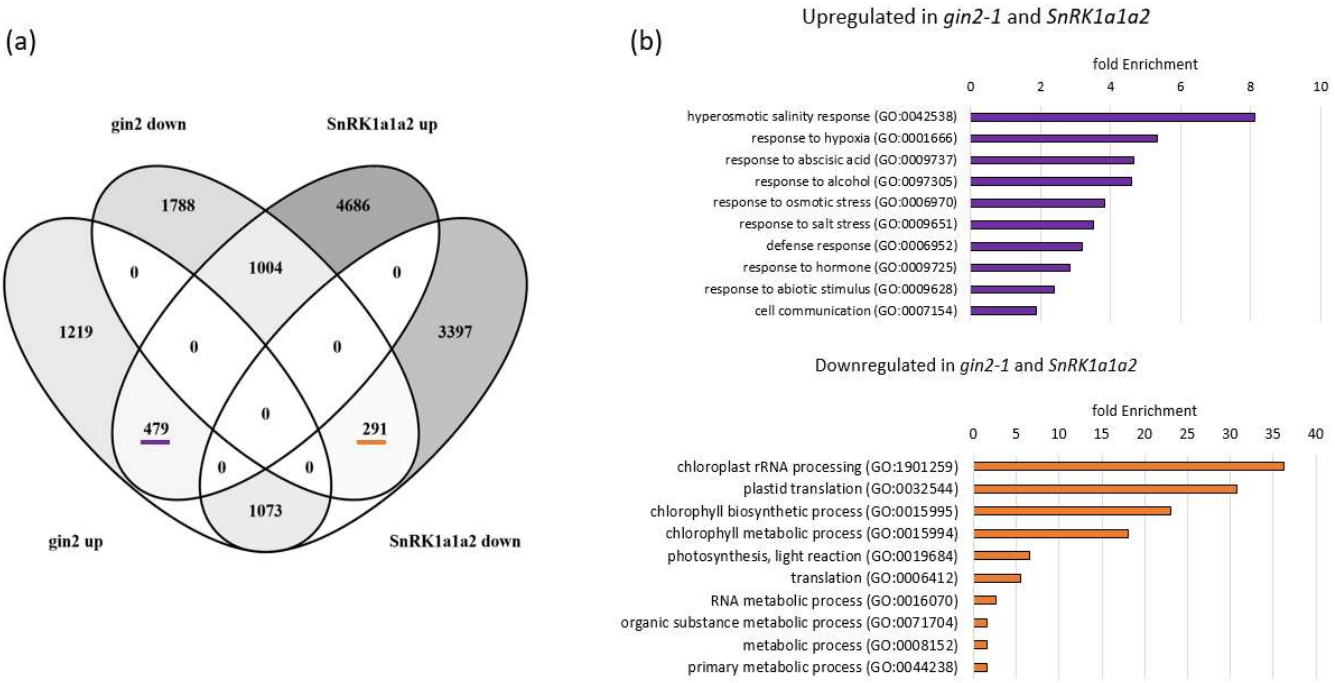

Fig. 3.4 Transcriptomic Overlap of *gin2-1* and *SnRK1a1a2*. Transcriptomic profiles from etiolated *gin2-1* cross-referenced to etiolated *SnRK1a1a2* seedlings from Henninger et al (2021). (a) Venn diagram showing counts for overlapping and uniquely regulated genes, generated using Venny 2.1 (*Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.* (https://bioinfogp.cnb.csic.es/tools/venny/index.html). (b) GO biological process terms for genes synergistically regulated by *gin2-1* and *SnRK1a1a2* either both upregulated (above, purple) or downregulated (below, orange).

Genes upregulated in both mutant transcriptomes show strong enrichment in response to various stress signal pathways, such as organic defence, hypoxia response, salt stress, and the abscisic acid (ABA) response pathway, which is induced by drought stress (Mukherjeeet et al, 2023). In particular, some genes of interest we can observe in this cluster are: *ABI1* (GO:0009737), which has been shown to negatively regulate protein kinases and other components in the ABA pathway (Vlad et al, 2009), *HFR1* (GO:0009737), a critical component in the perception of red/far-red light shade responses (Garcia and Rodriguez-Concepcion, 2023), *TOC1*, a core circadian oscillator gene (Millar et al, 1995), and *YUCCA8* (GO:0009725), an important gene in the auxin response pathway (Sun et al, 2012) (Fig 3.5).

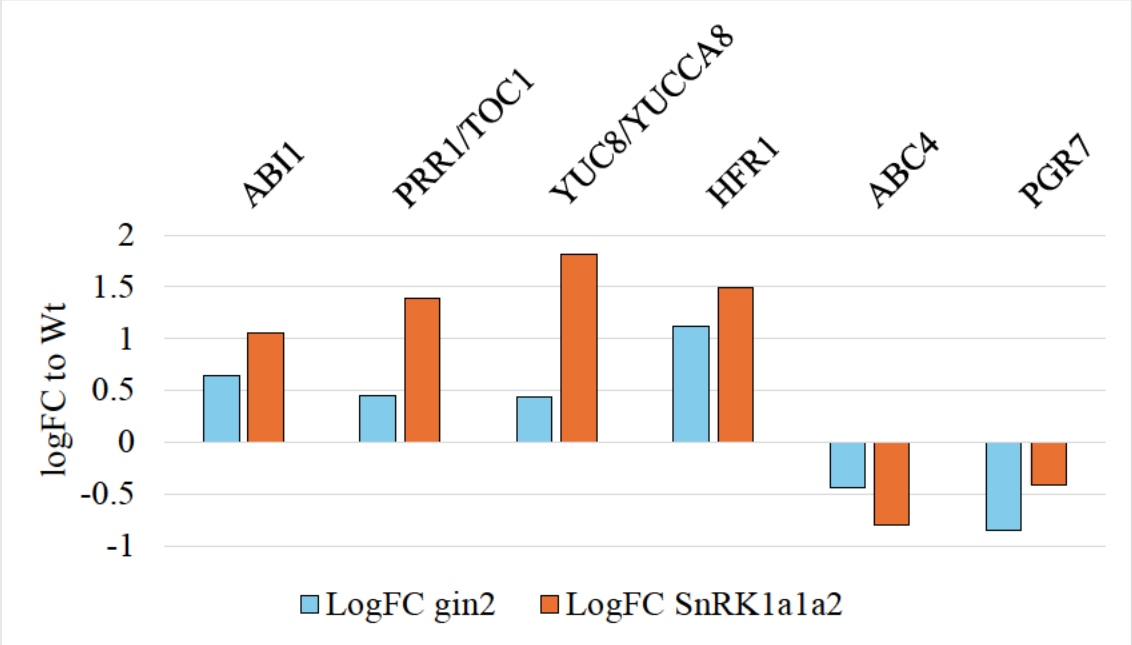

Fig. 3.5 Genes Misregulated in *gin2-1* and *SnRK1a1a2* in the Same Way Show Enrichment In Hormone Signal Pathways. Plotted logFC from *gin2-1* and *SnRK1a1a2* to Wt for several genes of interest.

Genes downregulated in both *gin2-1* and *SnRK1a1a2* show significant enrichment in chloroplast-associated processes, including the regulation of photosynthesis itself and well as control over the plastid genome. However, the majority of the genes in this cluster are, at this moment in time, largely undescribed. Some genes of note in this cluster are *ABC4* (GO:0019684), who's disruption has been shown to severely reduce the structural integrity of photosystem II (Shimada et al, 2005) and *PGR7* (GO:0019684), an essential component of electron transport in photosynthesis (Jung et al, 2010).

Given the significance of cross-regulation extends beyond what is regulated in the same direction, we wanted to dive further into the antagonistically regulated genes between the two datasets (Fig. 3.6). These clusters represent a zone of particular interest in the sense that HXK1-G6P activity is directly inhibitory to SnRK1 kinase activity, and as such we should expect to see significant enrichment in key metabolic processes here.

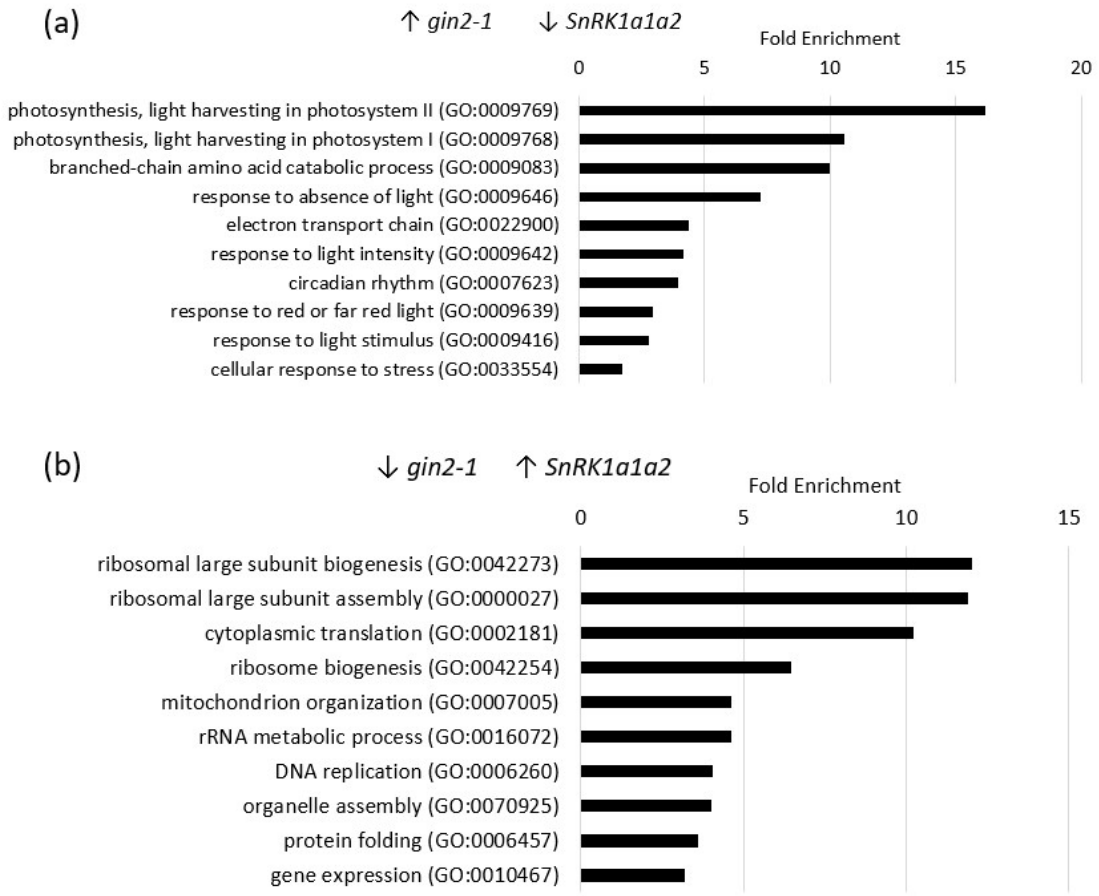

Fig. 3.6 Genes Opposingly Misregulated in *gin2-1* and *SnRK1a1a2*. Represents the gene ontology terms enriched in both *gin2-1* and *SnRK1a1a2* transcriptomes. (a) Genes up in *gin2-1* and down in *SnRK1a1a2.* (b) Genes down in *gin2-1* and up in *SnRK1a1a2*

Genes downregulated in *gin2-1* and upregulated in *SnRK1a1a2* (a cluster of 1073 genes) are highly enriched in RNA processing and ribosomal functions, providing interesting new insights into the mechanisms behind translational dynamics in response to sugar; interestingly, these translationally relevant genes do not extend to plastid ribosomal genes which are downregulated by both *gin2-1* and *SnRK1a1a2*. In this cluster, we can note the presence of ribosomal subunit proteins *RPL6A* and *RPP0C* (GO:0002181), *RPL18aD* (GO:0006416), and *TIM10* (GO:0007005). Genes downregulated in *SnRK1a1a2* and upregulated in *gin2-1* (a cluster of 1004 genes) show enrichment in photosynthetic pathways, BCAA catabolism, and red-light sensitivity. In this cluster, some genes of particular interest emerge, including the BCAA component genes (GO:0009083), *PIF4* and *PIF5* (GO:0010161), *CAB2* (GO: 0009768) and *ASN1* (GO: 0009063), and *KING1* (Fig. 3.7).

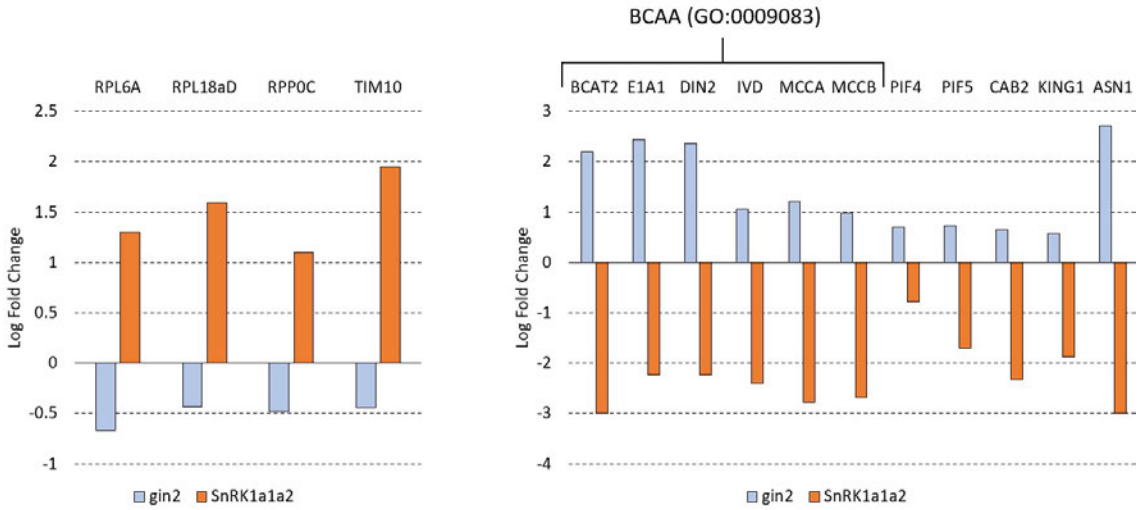

Fig. 3.7 Genes Opposingly Misregulated in *gin2-1* and *SnRK1a1a2* Involved in Ribosome, Starvation, and Light Signaling. Plotted logFC from *gin2-1* and *SnRK1a1a2* to Wt for several genes of interest. Genes on the left graph are associated with ribosomal subunits. BCAA=branched chain amino acid catabolism genes.

To summarize, we see opposing regulation of genes involved in photosynthesis, alternative metabolism, translation, and light sensing.

3.5 PIF and HXK1 Crosstalk Analysis

In the previous chapter of this thesis, we took a brief look at the cross-regulation of hypocotyl growth between *pifQ* and *hxk1-3*, taking particular note at the relevance of the enzymatic role in this overlap. To fully understand the breadth of the interregulation of growth between these two genes however, we wanted to explore the similarities and differences between transcriptomes for these two mutations.

We found a comprehensive RNA-seq dataset done on etiolated *pifQ* seedlings, and this was the best place to start our analysis (Zhang et al, 2013). This study described 2025 unique genes displaying a statistically significant two-fold misregulation by the *pifQ* mutation. In this transcriptome, we see strong enrichment in upregulation of genes involved in photosynthesis, the cell cycle and division, and translation.

This pattern is indicative of starvation, where significant resources are being diverted to increase the photosynthetic output. On the other hand, genes downregulated in *pifQ* show significant enrichment in the expected response to red light, as they are missing key red-light response genes, but also amino acid catabolism, hormone response, and the synthesis and breakdown of RNAs, which is consistent with a plant struggling to grow in the darkness.

Some genes of particular interest in the upregulated cluster are cell cycle genes such as *AUR1, ASE1,* and multiple cyclin kinases core to the cell cycle (van Leene et al, 2010).

We also see increased expression of *QQS*, an important orphan gene that modulates carbon/nitrogen storage and allocation (Wang et al, 2023), and key HXK1 responsive genes in *CAB1* and *CAB2*, commonly used as marker genes for HXK1-mediated signaling (Moore et al, 2003; Cho et al, 2006). In the downregulated genes, we observed significant misregulation in multiple auxin-responsive *SAUR* genes, as well as ABA-sensitive genes like *GBF3*. We also noted the presence of all BCAA genes previously described in the *gin2-1* RNA-seq analysis. Given the significance of their presence in the *gin2-1* transcriptome, coupled with other interesting genes of note, we wanted to take a closer look and break down the areas of overlap between *pifQ* and *gin2-1* transcriptomes (Fig. 3.8a). Due to the ineffectiveness of G6P on rescuing the *pifQ* hypocotyl phenotype, we wanted to only observe the most significant genes being misregulated between the two mutants, and thus chose a cutoff of logFC>0.4 or <-0.4.

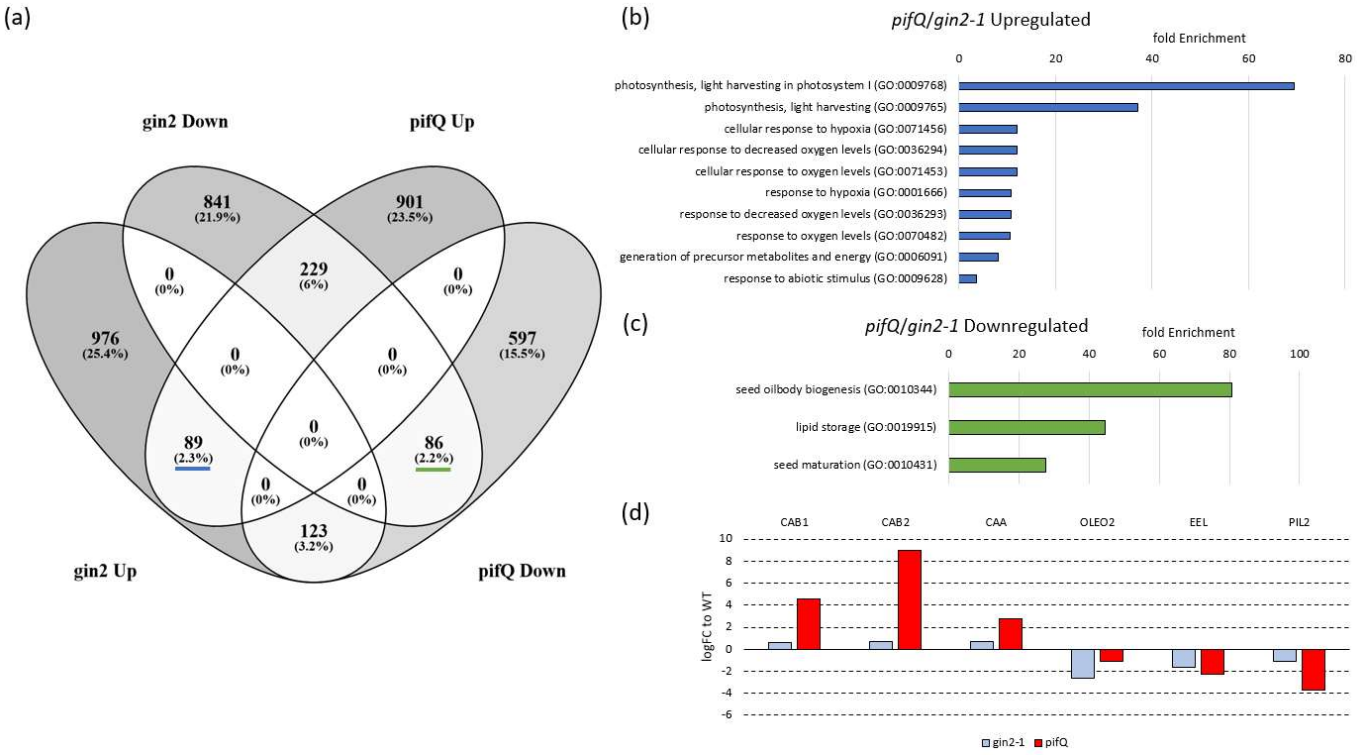

Fig. 3.8 Transcriptomic Overlap of *gin2-1* and *pifQ*. Transcriptomic profiles from etiolated *gin2-1* seedlings cross-referenced to *pifQ* seedlings from Zhang et al (2013). (a) Venn diagram showing counts for overlapping and uniquely regulated genes, generated using Venny 2.1 (*Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.* https://bioinfogp.cnb.csic.es/tools/venny/index.html). (b) GO biological process terms for genes synergistically upregulated in *gin2-1* and *pifQ (*c) GO biological process terms for genes synergistically downregulated in *gin2-1* and *pifQ* (d) logFC for genes of interest misregulated in both *gin2-1* and *pifQ. gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0

Of the 3,842 genes described between the two datasets, 527 were identified as being significantly misregulated in both *gin2-1* and *pifQ*, or approximately 13.7% of surveyed genes. Of these genes, 33% showed misregulation in the same direction and the remaining 67% were misregulated in opposing directions. Genes upregulated in both mutant lines showed enrichment in photosynthesis and hypoxia responsive processes (Fig. 3.8b); in this cluster we observed the genes *CAB1, CAB2,* and *CAA*, genes strongly associated with sugar signaling and the control of photosynthesis and response to sugar (GO:0009765) (Moore et al, 2003) (Fig 3.8c). Genes downregulated in both mutants shows a much more concentrated clustering, with very strong enrichment in lipid management and seed development (Fig 3.11c). In this cluster, we note the representation of *OLEO2* (GO:0010344), a gene highly

involved in oilbody formation in seedlings, as well as key embryonic transcription factors *EEL* and *PIL2* (GO:0010431) (Fig. 3.8d).

The two clusters with opposing directions of regulation are much more robust than the cooperative ones (Fig. 3.9).

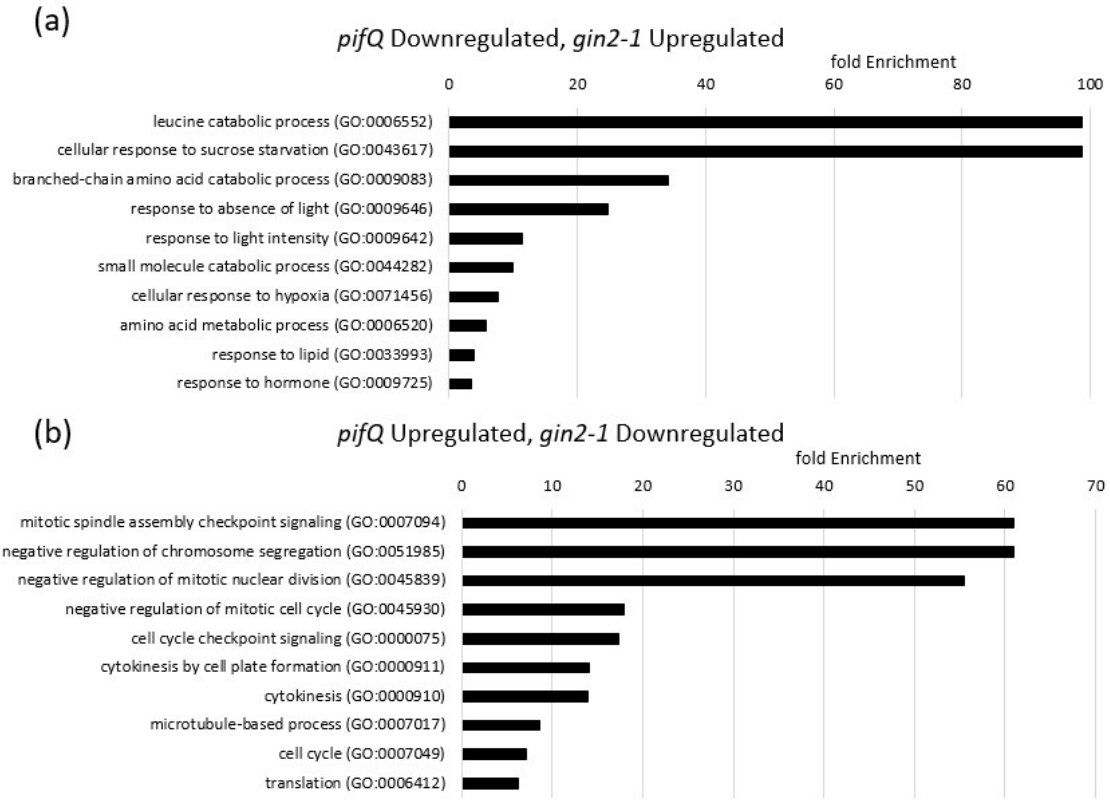

Fig. 3.9 Genes Opposingly Misregulated in *gin2-1* and *pifQ*. Represents the gene ontology terms enriched in both *gin2-1* and *pifQ* transcriptomes. (a) Genes up in *gin2-1* and down in *SnRK1a1a2.* (b) Genes down in *gin2-1* and up in *pifQ. gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0

Genes upregulated in *pifQ* but downregulated in *gin2-1* show significant enrichment in the cell cycle processes we'd seen represented in the *pifQ* dataset before; *AUR1* (GO:0007094) and the cyclin-dependent kinases (GO:0045839) previously mentioned are all seen in this cluster (Fig 3.14). In the opposite cluster, we see very strong enrichment in alternative metabolism, and in particular we note the presence of the BCAA catabolic processes represented here, as well as responses to light and darkness, hormones, and other abiotic stresses. In a closer look at the individual genes in this cluster, we can identify all previously

described BCAA genes (*IVD* not plotted for sake of scale, -26.72x in *pifQ* and 1.06x in *gin2-1*) as well as key red-light response genes *HFR1* and *PIL6* (Fig 3.10).

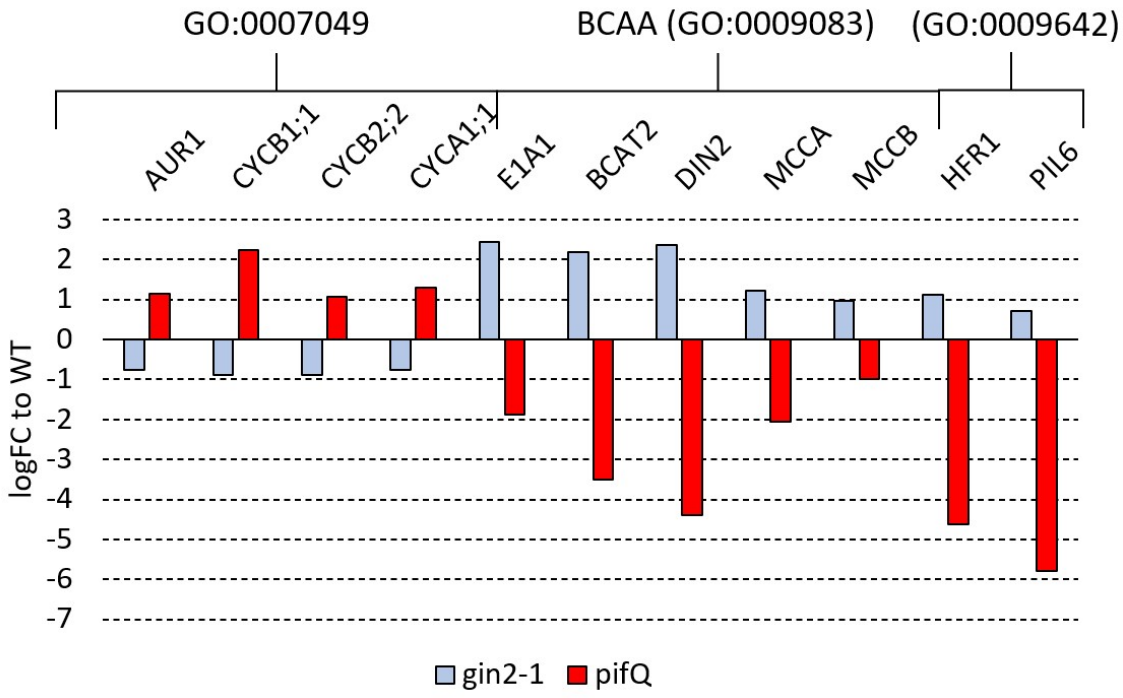

Fig. 3.10 Plotted Genes of Interest in *gin2-1* and *pifQ* Involved In Cell Cycle, Starvation, and Light. LogFC for genes antagonistically misregulated in *gin2-1* and *pifQ*. GO:0007069=cell cycle. BCAA=branched chain amino acid catabolism genes, GO:0009642=response to light. *gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0

3.6 SnRK1, PIF, and HXK1 Overlap

Now that we have explored the individual overlaps between HXK1, PIF, and SnRK1 transcriptomics, in order to fully understand the greater picture of the interregulatory network between these three pathways we wanted to examine the overlapping genes between PIF and SnRK1, and then compare that overlap to genes misregulated in *gin2-1*. Between *pifQ* and *SnRK1a1a2*, we can note a sizeable overlap in the transcriptome; with a cutoff of logFC $\geq$ 2 or logFC $\leq$ -2, we observed 523 genes misregulated in both datasets (Fig. 3.11)

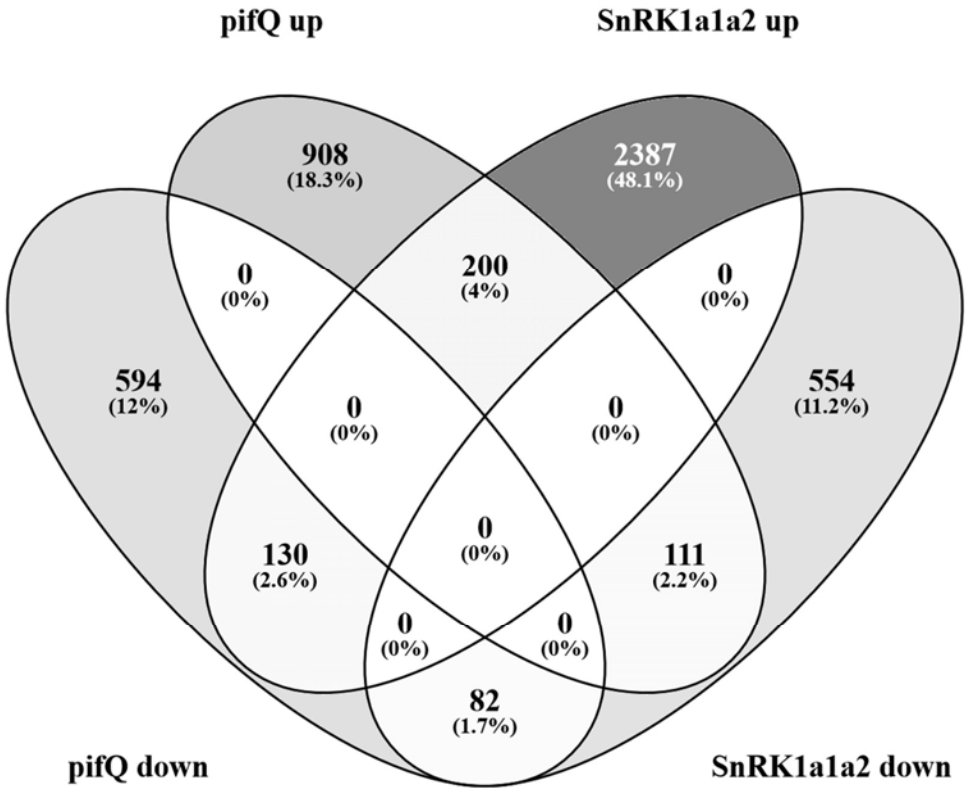

Fig. 3.11 Transcriptomic Overlap of *SnRK1a1a2* and *pifQ*. Transcriptomic profiles from etiolated *SnRK1a1a2* seedlings from Henninger et al (2021) cross-referenced to *pifQ* seedlings from Zhang et al (2013). Generated using Venny 2.1. *gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0. *SnRK1a1a2*= double mutant for two SnRK1a genes.

In these clusters, we saw the highest representation in genes upregulated in both mutant alleles (38%), and conversely the smallest representation in genes mutually downregulated (16%). To understand the significance of these clusters, we then looked at the enrichment factors here as well (Fig. 3.12). For clarity in the results, the clusters will be named off of the letter designation in the figure.

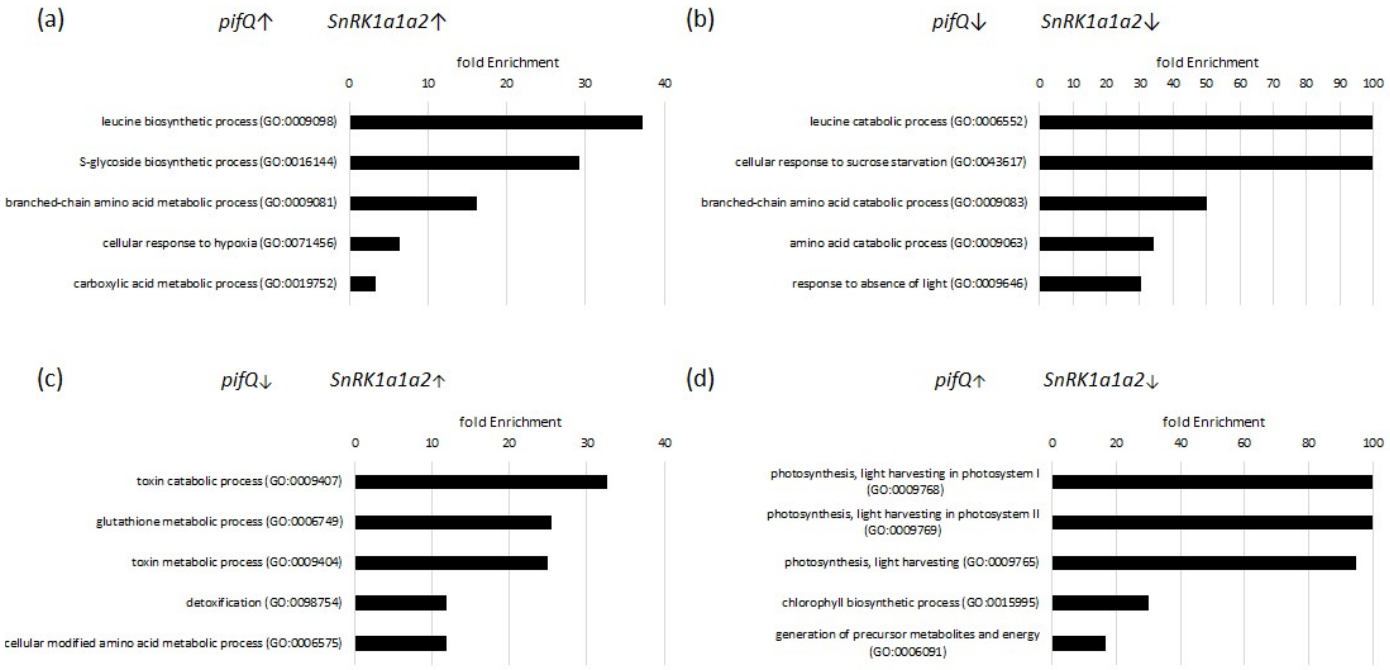

Fig. 3.12 Enrichment of Gene Clusters Transcriptomic Overlap of *SnRK1a1a2* and *pifQ*. Plotted fold enrichment for genes that are: (a) upregulated in *pifQ* and *SnRK1a1a2,* (b) downregulated in *pifQ* and *SnRK1a1a2,* (c) downregulated in *pifQ* and upregulated in *SnRK1a1a2*, and (d) upregulated in *pifQ* and downregulated in *SnRK1a1a2*. *gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0. *SnRK1a1a2*= double mutant for two SnRK1a genes.

In cluster (a), we see enrichment in various synthetic processes and hypoxia response genes, as well as a sizeable representation of genes involved in BCAA metabolism. As expected, in cluster (b) we see the opposite, where catabolic processes, including BCAA catabolism, are enriched, as well as starvation marker genes and etiolation factors. These results demonstrate significant coordination between the mechanisms behind metabolic adaptation to starvation and etiolation. In the other two opposing clusters, we see something slightly different. In cluster (c), genes involved in breakdown of toxins are downregulated in *pifQ* but upregulated in *SnRK1a1a2*, and finally in cluster (d) we see representation of photosynthetic genes being upregulated in *pifQ* and downregulated in *SnRK1*, as well as other chloroplast-relevant

processes like chlorophyll synthesis. To further explore the nature of these intersections, we wanted to take a look at some of the GO processes we've described throughout this chapter and how they are regulated between all three transcriptomes (Fig. 3.13).

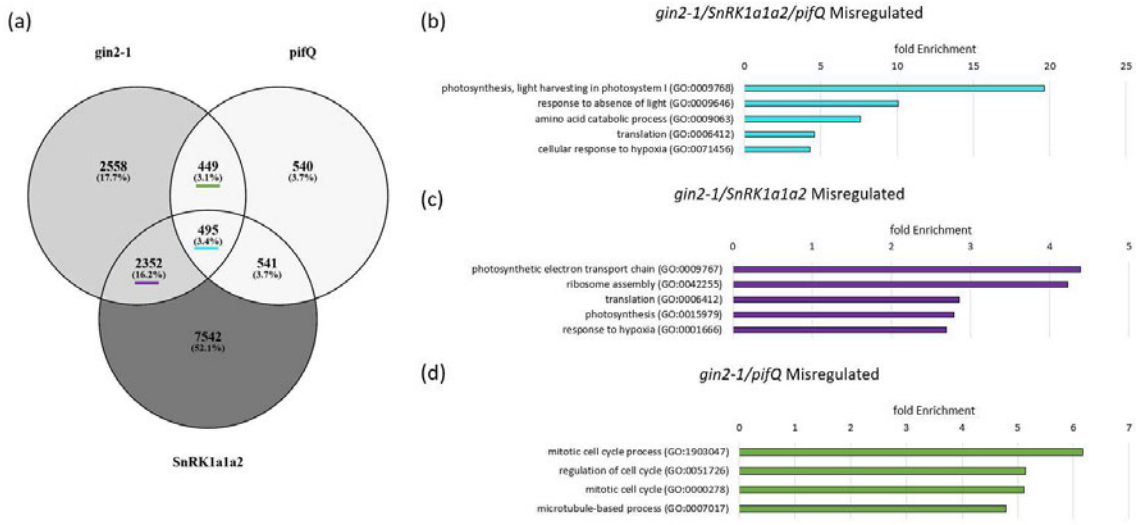

Fig. 3.13 Transcriptomic Overlap of *gin2-1, SnRK1a1a2,* and *pifQ*. Transcriptomic profiles from etiolated *gin2-1* seedlings, *SnRK1a1a2* seedlings from Henninger et al (2021), *pifQ* seedlings from Zhang et al (2013). (a) Venn diagram (Venny 2.1) showing counts in each overlap. (b) GO term enrichment for genes misregulated in all three datasets. (c) GO term enrichment for genes misregulated in *gin2-1* and *SnRK1a1a2* not found in *pifQ*. (d) GO term enrichment for genes misregulated in *gin2-1* and *pifQ* not found in *SnRK1a1a2*. *gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0. *SnRK1a1a2*= double mutant for two SnRK1a genes.

In all three mutant transcriptomes, there is significant enrichment in genes associated with photosynthesis, amino acid catabolism, and translation (Fig. 3.13b). However, upon closer examination of the overlap regions, we can see that all these processes are further represented within the *gin2-1/SnRK1a1a2* intersection (Fig. 3.13c) and show no significant enrichment in the *gin2-1*/*pifQ* overlap (Fig. 3.13d). In that cluster, we detect almost exclusively genes associated with the cell cycle. Thus, we wanted to plot some of the genes we have been examining throughout this chapter to observe their specific regulations (Fig. 3.14)

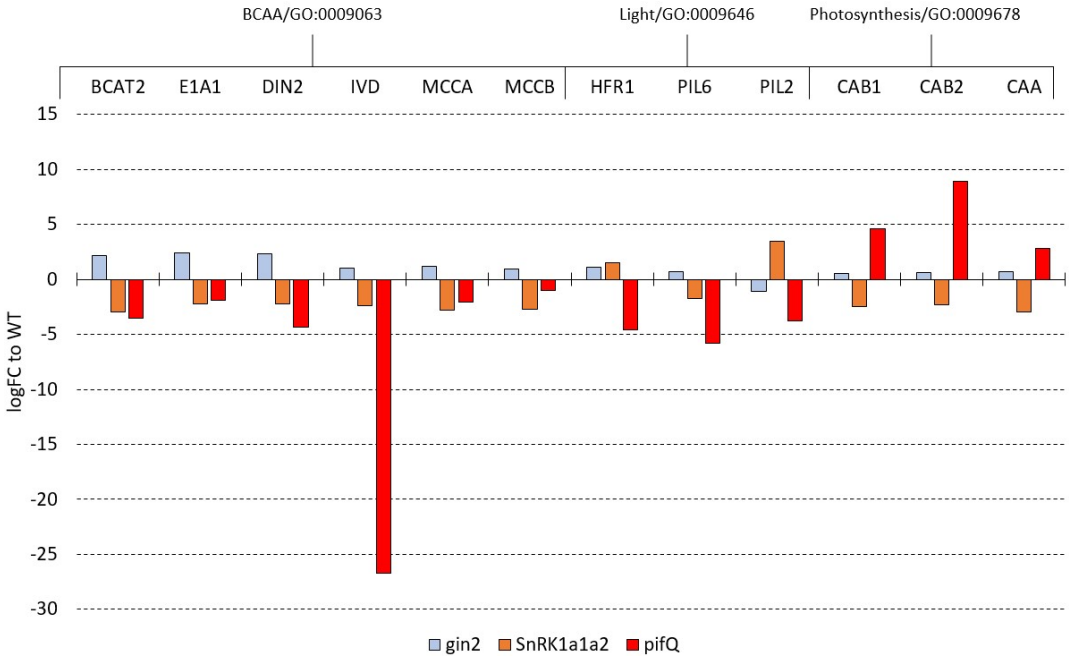

Fig. 3.14 Genes of Interest Regulated in *gin2-1, pifQ,* and *SnRK1a1a2* Involved In Starvation, Light, and Photosynthesis. Plotted logFC of genes present in all three analysed transcriptomes from figure 3.13b. BCAA=branched chain amino acid catabolism. *gin2-1*=HXK1-deficient mutant in Ler. *pifQ*=quadruple mutant for PIF1, PIF3, PIF4, and PIF5 genes in Col-0. *SnRK1a1a2*= double mutant for two SnRK1a genes.

All three mutant alleles show regulation of the BCAA genes, further cementing their importance at the nexus of light and sugar signaling as seen throughout this chapter. The incidence of several red-light associated genes is consistent with the understanding of metabolic and red-light signaling overlap. Curiously, *PIL6/PIF5* is the only gene in this particular group with regulation in the same direction between *SnRK1a1a2* and *pifQ*, possibly due to its interactions with SnRK1 protein (Hwang et al, 2019). Additionally, the presence of photosynthesis genes *CAB1/2* and *CAA* in all three transcriptomes also alludes to the significance of HXK1 regulation of the plastome. Notably absent from the overlapping genes are the cyclin-dependent kinases and other cell-cycle genes previously highlighted in figure 3.10.

3.7 Discussion

HXK1 has been prominently studied throughout the past few decades in regard to its role as a sugar signaling molecule (Moore et al, 2003, Cho et al, 2006, Bruggeman et al, 2015 van Dingenen et al, 2021); given the significance of its role in developing seedlings as described in chapter 2 of this thesis, it is important to re-examine the processes that are affected by HXK1 by closely examining and comparing available transcriptomic datasets. This in turn provides additional interpretations on older works that have explored the significance of HXK activity in development, as we can begin to shape potential regulatory networks between pathways to direct future endeavours with increased clarity on the outset.

In our transcriptomic analysis of the etiolated *gin2-1* seedling, we noted that there was a sizeable representation of plastome genes, particularly in the chloroplast where nearly all of the genome is misregulated by the *gin2-1* mutation. In dark-grown seedlings, we observed an increase in the expression of 82.8% of chloroplast genes and 23.5% of mitochondrial genes in the *gin2-1* mutant. Although previous studies have demonstrated that glucose treatment can mitigate chlorophyll degradation in darkness, our findings suggest that HXK1 suppresses chloroplast gene expression during dark conditions, consequently leading to reduced chlorophyll levels upon exposure to light (Ueda et al., 2020). The established link between chloroplast biogenesis and metabolism is further supported by observations in albino mutants, which exhibit significant dysregulation in genes associated with energy starvation and mobilization, indicating a transition in growth strategy towards autotrophism (Grübler et al., 2017). Regarding mitochondria, sucrose deprivation induces structural alterations that impair protein synthesis despite unaffected transcriptional activity (Giegé et al., 2005). Moreover, our study demonstrates that HXK1 downregulates the expression of mitochondrial genes involved in various processes such as electron transport, ATP synthesis, and oxidative responses. Mitochondrial oxidation is intricately linked to metabolism and plays a crucial role in maintaining signaling capacity during both abiotic and biotic stress responses (Vanlerberghe, 2013). The observed effects of the *gin2-1* mutation on organellar transcriptomes likely reflect the cellular response to carbon resource limitation, emphasizing the necessity to prepare the cellular machinery for the transition to photosynthesis rather than relying solely on seed reserves for growth fuel.

Analysis of the previously performed RNA-seq experiment on the nuclear genome shows high enrichment in alternative metabolic pathways in the upregulated genes, consistent with

the findings from chapter 2 which suggest HXK1's role in the seedling is primarily as an enzyme; its loss results in increased activity in non-glucose metabolism, which can similarly be observed in other metabolic mutant lines where there is an increased abundance in alternative metabolites, including BCAA intermediates that's production is dependent upon genes which were highlighted in our data (Nukarinen et al, 2016). BCAA-associated proteins are consistently interrogated by multiple studies when investigating sugar starvation in *Arabidopsis* (Fujiki et al, 2001; Hirota et al, 2018; Pedrotti et al, 2018). Pedrotti et al. highlights the extent at which SnRK1 is involved in the activation of alternative metabolic pathways in response to sucrose starvation (as a result of extended night and deprivation of photosynthetic reserves), and so we wanted to closely observe the other processes that are similarly regulated between SnRK1 and HXK1 via their transcriptomic dataset and ours.

In this, we noted that HXK1 and SnRK1 both regulated suites of genes that were indicative of a shift in the plant's shifting priorities in response to perceived starvation from a loss of function in key metabolic signal genes, which is expected to observe given their respective roles in normal metabolic function. It was then pertinent to observe which genes were not affected by SnRK1 and only HXK1, which could imply a regulatory pathway distinct of HXK1's control over metabolism via glucose phosphorylation, and thus potentially owing to its signaling role or through an undescribed alternative G6P-induced signal pathway independent of SnRK1. In this cluster, we noted a high percentage of the plastid genome (47% of the chloroplast, 30% of the mitochondria); interestingly, we discovered that the RPO family genes, master regulators of the chloroplast transcriptome (Liebers et al, 2018; Tadini et al, 2020), were in this particular cluster; this suggests that HXK1 holds strong influence over the plastome independently of SnRK1. ABI4 is also in this cluster, and given it's published reputation as a sugar signaling response gene this suggests that HXK1 is responsible for this activity, and bridges sugar signaling to other ABI-regulated responses via HXK1 as a signal transducer (Bossi et al, 2009).

While it is important to understand the likewise relationship between genes, in this particular case it becomes more interesting to understand the antagonistic relationship between HXK1 and SnRK1; HXK1 directly affects the function of SnRK1 through the production of trehalose-6-phosphate (Tre6P) being dependent upon G6P abundance. Tre6P is well characterised as a negative regulator of SnRK1 through the inhibition of the KIN10 subunit of SnRK1, repressing its activity (Zhang et al, 2009). In the antagonistic clusters, we can see strong representation of key signaling processes in either direction of regulation. HXK1

shows significant upregulation of ribosomal and translational machinery, while SnRK1 shows the opposite response. Metabolic influence on translation has been well characterized in *Arabidopsis*, with a particular focus on the role of TARGET OF RAPAMYCIN (TOR) in response to starvation, where it significantly stimulates ribosome biogenesis and mRNA translation (Haq et al, 2022). TOR was not represented in the *gin2-1* mutant transcriptome, though has been shown to be activated by the presence of glucose (Xiang et al, 2013; Li et al, 2017). It is then possible that the antagonistic relationships between HXK1 and SnRK1 might be explained by HXK1 interactions with TOR in the cytoplasm; TOR and SnRK1 have been demonstrated to have antagonistic functions in nutrient sensing, control of metabolism, and regulation of certain genes (Shi et al., 2018; Baena-González and Hanson, 2017), and in *Arabidopsis* SnRK1-Tre6P shows significant transcriptional control over multiple photosynthetic genes (Zhang et al, 2009).

In the other direction, we can note that HXK1 exhibits negative control over photosynthesis, light signaling, and BCAA catabolism, while SnRK1 promotes these processes. In *Arabidopsis*, SnRK1 regulation of red light signaling is relatively undescribed, though a study done on tomato seeds demonstrates some connection between the two as SNF4 and SNF1, SnRK1 analogues in tomato, show increased expression in far-red light (Bradford et al, 2003). Conversely, the importance of SnRK1 on photosynthesis is somewhat characterized; in tomato, SnRK overexpression induces chlorophyll synthesis and the expression of photosynthetically relevant genes (Liang et al, 2021). When taken together, the antagonistically regulated genes between HXK1 and SnRK1 demonstrate the nuances of metabolically regulated processes and the potential importance of Tre6P production (dependent on HXK1 activity) on the biological processes discussed here.

Another pathway that intersects significantly with ABI is red light signaling mediated via PHYs and PIFs (Liang et al, 2020; Li et al, 2022). Additionally, due to the phenotypic similarities between etiolated *pifQ* and *gin2-1* seedlings described in the previous chapter of this thesis, we hoped an analysis of *pifQ* transcriptome would provide further clarity into the nature of this overlap as *pifQ* seedlings were insensitive to G6P treatment. In this analysis, we discovered that *gin2-1* and *pifQ* seedlings showed enrichment in genes involved in photosynthesis and seed storage in a similar way. In particular, the cross-regulation of important sugar-responsive *CAB1/2* and *CAA* genes indicate more nuance in the potential cross-regulatory capacity of HXK1 and PIFs. Insofar as we could discern, little work has been done on the regulation of *CAB* via PIFs; *TIMING OF CAB 1* (*TOC1*), a circadian

oscillator gene that influences the expression of *CAB2*, has been shown to directly bind to and inhibit the repression of certain genes via PIF3 and 4 activity (Soy et al, 2016; Zhu et al, 2016). It is possible that the regulation of *CAB2* via PIFs may be done through the regulation of *HXK1* expression; ChIP-seq analysis of PIF3 binding sites did not definitively show attachment near the promoter of *CAB2* (Zhang et al, 2013), while HXK1 has been demonstrated to bind to the promoter of this gene (Cho et al, 2006). This would explain the similar regulation of *CAB1/2* and *CAA*.

However, the majority of overlap between the two transcriptomes is antagonistic, with high enrichment in cell cycle genes being downregulated by *gin2-1* and upregulated by *pifQ* and even greater enrichment in alternative metabolic pathways via BCAA catabolism. This is consistent with previous observations in chapter 2 on the lack of impact G6P treatment had on *pifQ* seedlings; if cell cycle genes are being suppressed in the *gin2-1* mutation, it would stand to reason that the endoreduplication process would also be impacted thus limiting the expansion of individual cells as well, which is consistent with previous observations linking *TOR* to this process (Kalve et al, 2014). We can see that G6P rescues this phenotype here, but in *pifQ* where the expression of these genes is elevated, G6P has little to no effect; other processes seemingly limiting the growth of the cells and seedlings in a sugar-independent manner. In this study, we did not quantify the cell profile of *pifQ* seedlings, however in rice it is understood that PIF homologs primarily regulate cell division rather than cell expansion (Ji et al, 2019). In our studies, HXK1 has been implicated in both cell division and cell expansion, and other studies have demonstrated this in adult tissues as well (Appendix A, van Dingenen et al, 2019). However, while we have demonstrated the dependence on G6P of cell expansion in *gin2-1,* we have not quantified the effect of G6P on the count of cells in the hypocotyl. It is then possible that the effect of HXK1 on cell division is independent of its ability to produce G6P, thus explaining the overlap of PIF and HXK1 on growth being independent of G6P production. However, as previously mentioned in chapter 2, *pifQ* is a quadruple mutant for *PIF1/3/4/5*, and it is possible that other PIFs may be more directly tied to HXK1-G6P in the mediation of growth.

In light of PIF independence on G6P from hypocotyl elongation, we wanted to further explore alternative points of interaction between PIFs and metabolic signaling, and thus began an investigation into potential cross-regulation revealed in the *pifQ* and *SnRK1a1a2* transcriptomes described in this chapter. In terms of sheer number of genes cross-regulated,

between *pifQ* and *gin2-1* we counted 527 genes, while *SnRK1a1a2* and *pifQ* shared a total of 523 genes across the four clusters of overlap. However, the relationship between the two transcriptomes showed different directions of regulation in the processes enriched in the overlap between them and *gin2-1* independently. Photosynthetically relevant genes *CAB1/2* and CAA were upregulated in both *gin2-1* and *pifQ* yet downregulated in *SnRK1a1a2*, which is consistent with previous observations on the nature of HXK1-G6P on SnRK1 activity. However, the BCAA genes were synchronously downregulated by both *SnRK1* and *pifQ*, while being up in *gin2-1*, suggesting that the increase in alternative metabolites is independently regulated between PIFs and HXK1-G6P-SnRK1. We can thus infer through the nature of these interactions that a key point of intersection between sugar and light signaling resolves at the control of photosynthesis through the control of chloroplast synthesis and modifications on photosystem activity. To further test the impact of this cross-regulation, it would be pertinent to monitor the greening capacity (via chlorophyll accumulation) of *pifQ*, *SnRK1*, and cross-bred seedlings of these mutants both with and without G6P as well as a competitive HXK inhibitor. *pif1, pif3*, and *pifQ* have been demonstrated to have significantly reduced greening rate, with *pifQ* almost unable to green when transferred from the dark to the light (Chen et al, 2013). It would then be interesting to determine if this process could be rescued through exogenous G6P in a way that seedling growth could not. The absence of cell cycle genes from the *SnRK1a1a2* transcriptome while present in both *gin2-1*and *pifQ* gives further support for our emergent hypothesis on how PIFs and HXK1 resolve to cross-regulate growth independently of G6P activity, as G6P has direct implications on SnRK1 activity and thus its role in development.

3.9 Conclusion

In conclusion, our investigation into the genetic and molecular underpinnings of the *gin2-1* mutant, particularly in the context of HXK1-G6P regulation of growth, has revealed insight into the regulatory networks governing plant growth and development. The comprehensive analysis of RNA-seq data highlighted a significant misregulation of 2344 genes in *gin2-1* compared to Wt, with distinct patterns of upregulation and downregulation. Notably, the upregulated genes were associated with a starvation-type response, cellular respiration, ATP biosynthesis, amino acid catabolism, and photosynthesis, indicative of a metabolic shift underpinning the mutant phenotype.

Further delving into the intersection of HXK1 and SnRK1 shed light on the intricate cross-regulatory processes between these key players in plant metabolism. The significant overlap in misregulated genes between *gin2-1* and *SnRK1a1a2* mutants suggests a coordinated response to metabolic signals. However, the identification of genes exclusively misregulated in *gin2-1*, particularly those associated with cell cycle regulation and plastid genes, unveils a unique role for HXK1 beyond its canonical metabolic functions. The presence of RPO family genes in this cluster implicates HXK1 in regulating the chloroplast transcriptome, emphasizing its multifaceted role in development.

By analysing the transcriptome of *pifQ* seedlings, we aimed to understand their insensitivity to G6P treatment and the underlying regulatory mechanisms that results in phenotypic similarity to *gin2-1* without its reliance on G6P for growth. Our analysis revealed similarly enriched genes related to photosynthesis and seed storage in both *gin2-1* and *pifQ* seedlings, suggesting cross-regulation between HXK1 and PIFs particularly in sugar-responsive genes like *CAB1*/*2* and *CAA*. However, the majority of overlap in transcriptomes shows antagonistic regulation, with cell cycle genes being downregulated in *gin2-1* and upregulated in *pifQ*, indicating potential impacts on cell expansion mediated through the TOR pathway. While PIFs are known to primarily regulate cell division, HXK1 influences both division and expansion, suggesting a complex interplay between these pathways in modulating plant growth and development. Further research is needed to elucidate the specific mechanisms underlying their interaction and its implications for plant physiology.

We further investigated alternative points of interaction between PIFs and metabolic signaling by analysing the *pifQ* and *SnRK1a1a2* transcriptomes. There were 527 genes cross-regulated between *pifQ* and *gin2-1*, while 523 genes overlapped between *SnRK1a1a2* and *pifQ* across four clusters. However, the regulatory directions differed between these transcriptomes and *gin2-1*. Genes related to photosynthesis, such as *CAB1*/2, *CAA*, and *ASN1*, were upregulated in *gin2-1* and *pifQ* but downregulated in *SnRK1a1a2*, consistent with HXK1-G6P's influence on SnRK1 activity. Conversely, BCAA genes were downregulated in both *SnRK1a1a2* and *pifQ* but upregulated *in gin2-1*, suggesting independent regulation of these genes between PIFs and HXK1-G6P-SnRK1. This indicates a critical intersection between sugar and light signaling at chloroplast synthesis and photosystem activity control. Monitoring the greening capacity of these seedlings with and without G6P or an HXK inhibitor could provide further insights into this cross-regulation's impact on plant growth

In conclusion, our study not only expands our understanding the mechanisms that determine the *gin2-1* mutant phenotype but also uncovers the multifaceted roles of HXK1 in orchestrating diverse cellular processes beyond its canonical metabolic functions. The cross-regulatory capacity between HXK1, SnRK1, and PIF signaling underscores the complexity of plant growth regulation, highlighting several important niches that these pathways converge at, such as photosynthesis and at the cell cycle. Future research endeavours should delve deeper into the molecular mechanisms governing these interactions, providing valuable insights for crop improvement and addressing challenges in agricultural sustainability; understanding the interplay between sugar status and quality of light can influence how vertical farming can be optimized for increased yield. As PIFs are also potent thermosensors, it also becomes relevant to study their transcriptomic profile and how it interacts with other pathways in this era of climate change (Casal and Fankhauser, 2023).

Chapter 4: HXK1 and PHYTOCHROME pathway convergence

4.1 Introduction

In chapter 2, we established a partial lack of coordination between the regulation of hypocotyl length via HXK1 and several PIFs by analysing the response of *pifQ* to G6P, to which it was insensitive to. However, as alluded to in the discussion, *pifQ* does not include a mutation in *PIF7*, which has been more recently described in detail as a chief component in regulating the response to far-red light, particularly at the end of the day where *pif7* mutant seedlings show little to no response to treatment (Leivar et al., 2008). PIF7 has a unique structure when compared to other PIF family members, with a long glutamine rich (poly-Q) sequence towards the N-terminus of the protein that it only shares with PIF8, which can potentially result in additional protein-protein interactions or further transcriptional activity (Leivar et al., 2008; Freiman and Tijan, 2002). Additionally, the phosphorylated form of PIF7 is not degraded by PHYB as PIF3, PIF4, and PIF5 are, but is retained in the cytosol through interaction with 14-3-3 proteins (Huang et al., 2018). These factors which set PIF7 apart from the others, coupled with its seemingly unique dominance over the end of day far red light (EoDFR) response (end of day far red is when 40µmol $m^{-2}s^{-1}$ of far-red (700-780nm) light is applied just before darkness), leads us to question if there is interaction between the PIF7 and HXK1 pathways in hypocotyl elongation; given the lack of association between other PIFs and HXK1 despite control over similar responses in development.

The EoDFR light response represents an important biological niche in neighbouring plant detection; throughout the day, growing plants may be shaded from direct sunlight while the sun is high in the sky, and as the incident of light lowers while the sun sets on the horizon, sunlight shining through neighbouring plants in enriched in far red light (Ballare, 1999; Kohnen et al, 2016; Roig-Villanova et al, 2019). This allows growing plants to modify their growth to outcompete their neighbours, inducing a shade avoidance syndrome (SAS), which is characterized by elongated shoots, reduced leaf area, induction of flowering, and changes in metabolic state that reflect this focus on growth (Halliday et al, 1994; Kim et al, 2008; Bou-Torrent et al, 2015; Ballare & Pierik, 2017; Morelli et al, 2021; Romanowski et al., 2021). It is important to note that while the *pif7* mutant shows a significantly reduced EoDFR response, only the *pif457* triple mutant completely loses its elongation response to shade, and

additional studies indicate that PIF7 activity may be dependent on PIF4, increasing the complexity of this response (de Wit et al, 2015; Fiorucci et al, 2020; Willige et al, 2021). Published RNA-seq data from our lab have shown that in response to EoDFR treatment all *HXK* family genes are significantly misregulated, showing strong suppression for the majority of the family when treatment is started in the late stages of seedling development and the onset of adult leaf development (Fig. 4.1a). Additionally, analysis of the *HXK1* promoter region reveals several binding sites that PIF7 is able to bind at and potentially regulate its transcription (Fig. 4.1b)

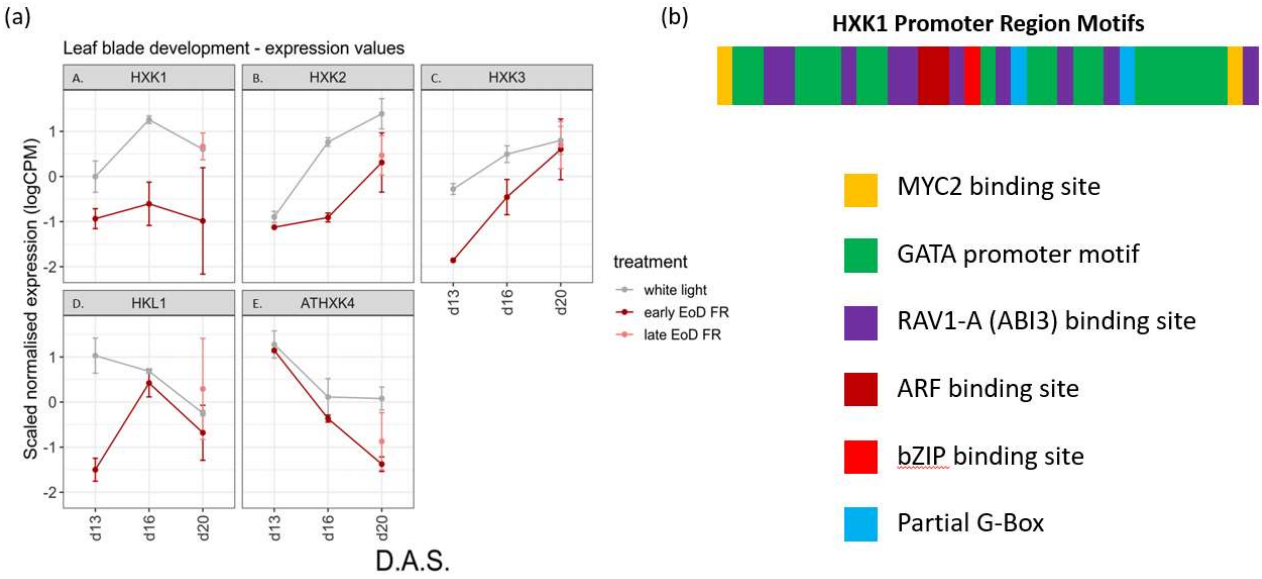

Fig. 4.1. RNA-seq Analysis of EoDFR Response Shows HXK Genes Implicated in Shade Avoidance. (a) Data from Romanowski et al (2021), expression of HXK family genes at various days after stratification in response to EoDFR applied at day 6, and EoDFR applied at day 18. Leaf 3 of the adult rosette was sampled at ZT 22 on days 13, 16, and 20. (b) Analysis of predicted HXK1 promoter region for canonical binding sites. PIF7 binds to G-box sites.

Given these developments, we see a potentially significant hole in our understanding of both PIF7 and HXK1 in seedling development, and here our investigation starts; our guiding hypothesis for this chapter was that PIF7 mediates growth in an EoDFR SAS by suppressing expression of *HXK1*.

4.2 PIF7 Regulates Expression of *HXK1* under End of Day Far Red Conditions

At this point, we do not understand the potential mechanisms by which PIF7 hypothetically regulates *HXK1* and other *HXK* family genes. PIFs have been demonstrated to regulate genes at both the transcriptional and protein level (Leivar and Monte, 2014), so it was important to test our guiding hypothesis. In order to test our hypothesis, we performed a basic RT-qPCR analysis of *pif7-1* seedlings with and without EoDFR treatment and observing the expression of each member of the *HXK* gene family (Fig. 4.2).

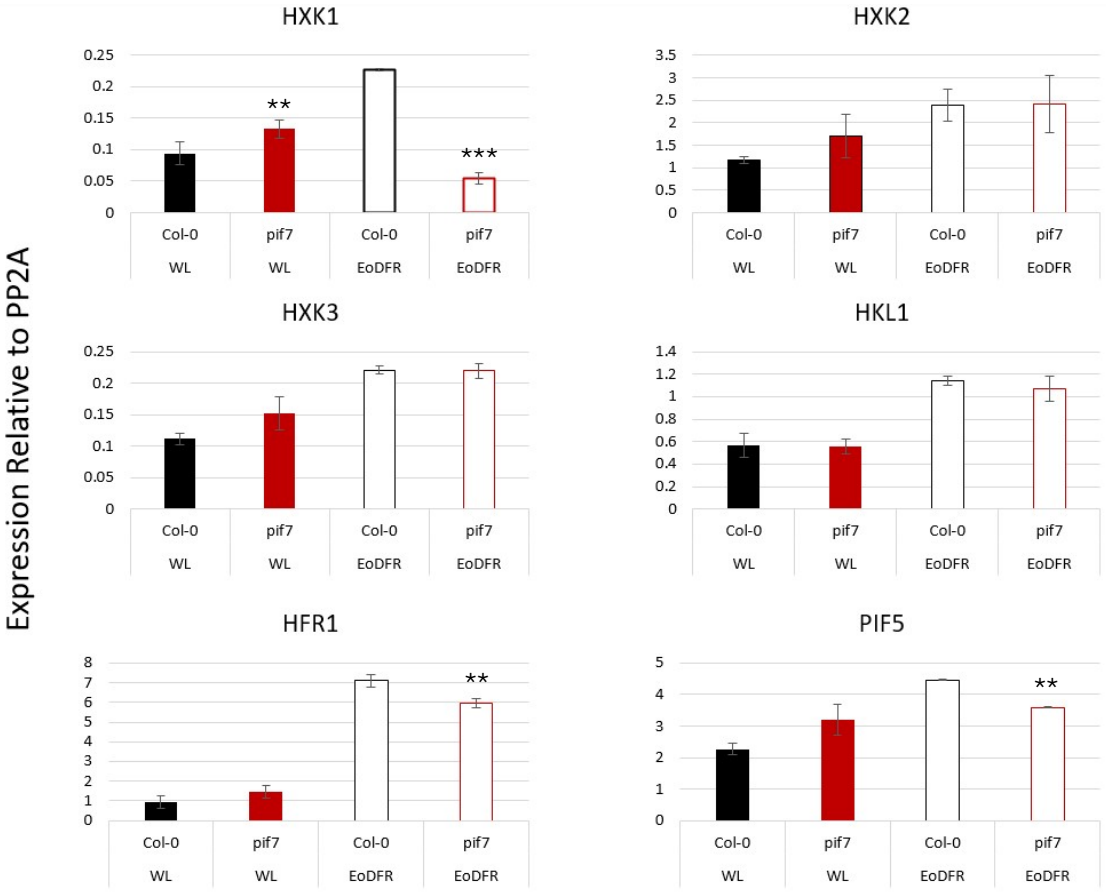

Fig 4.2 *pif7-1* qPCR Shows Significant Misregulation of HXK1. 4-day old 8:16 standard white light seedlings of Wt (black) and *pif7-1* (red) grown. Filled bars are grown without additional treatment, cleared bars are treated with 10 minutes of 40 µmol $m^{-2}s^{-1}$ far-red light after lights are turned off for the night after 1 day post stratification. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01, ***=pval<0.001. Col-0=Columbia (wild-type) *pif7*=PIF7-deficient mutant in Col-0.

Curiously, in this experiment we found that EoDFR induced expression of *HXK* family genes in Wt, opposite of what was demonstrated in the data described earlier from leaf tissue (this

experiment was done in seedlings). These results indicate that PIF7 uniquely regulates *HXK1* expression amongst the *HXK* family genes, and this regulation is most prominent in EoDFR conditions where it is not only required for the induction of *HXK1* expression but also alleviates a repressive effect. We attempted to confirm the validity of this misregulation by quantifying the expression of known FR regulated gene *LONG HYPOCOTYL IN FAR-RED* (*HFR1*) and PIF7-regulated *PIF5* which showed expected patterns of misregulation based on previous work, though it appears that according to our data this can only be minorly attributed to PIF7, though this is consistent with the published data taken around the time point observed (Leivar et al, 2020, Yang et al, 2023). This presents a challenge when interpreting data, as our positive controls proved unreliable in this experiment; further repeats with other, more reliable PIF7-dependent genes, such as ATHB2 described in Yang et al (2023) may prove useful in the interpretation of this data or taking samples at alternative time points (or even a full time-course assay). However, despite all the HXK family members showing increased expression in response to EoDFR, PIF7 appears to only be involved in the regulation of *HXK1*. This shows that PIF7 positively regulates *HXK1* expression in EoDFR and mildly negatively regulates it in WL, although the latter was not significant. To be entirely sure of the extent of the validity of these results, this experiment needs to be repeated, but in hand these results begin to shape our hypothesis.

4.3 *pif7-1* Responds to Exogenous G6P

Like other *pif* mutants, *pif7-1* exhibits a short hypocotyl phenotype under light-limiting conditions, as previously described (Leivar et al., 2020). Consistent with these results, we found that *pif7-1* mutant seedlings were significantly shorter than Wt plants under short-day and dark conditions. We wanted to test our hypothesis that G6P-mediated hypocotyl elongation is stimulated by PIF7 similarly to HXK1, and if so, we would expect to observe a rescue of the *pif7-1* short hypocotyl by applications of exogenous G6P. (Fig. 4.3).

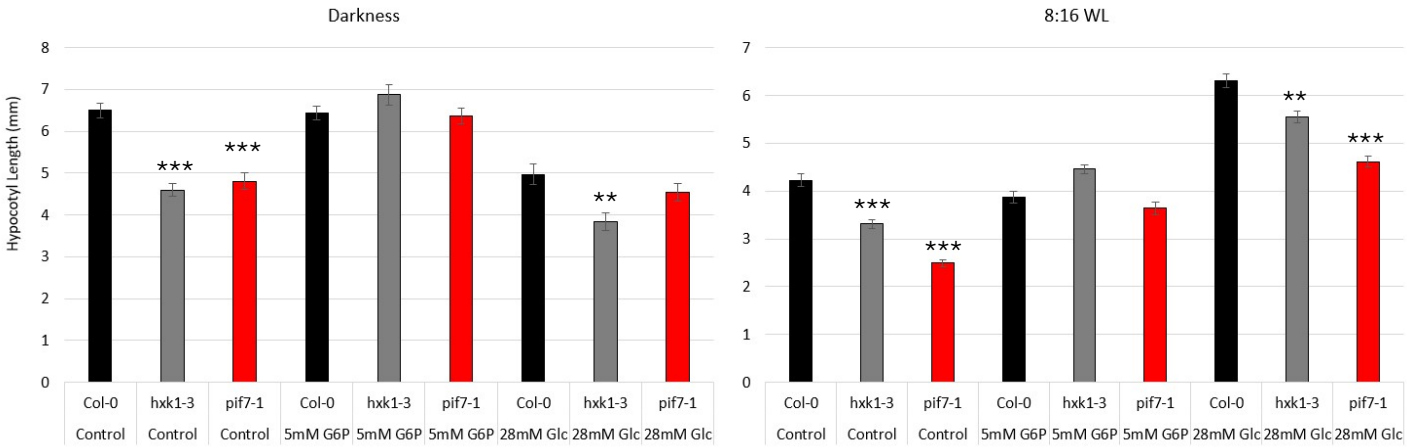

Fig. 4.3 Effects of Exogenous Sugars on *pif7-1* Growth Demonstrates G6P-dependent Growth Via PIF7. 4-day old seedlings of Wt (black), *hxk1-3* (grey), and *pif7-1* (red) grown in darkness (left) or in 8:16 standard WL (right). Seedlings grown on control, 5mM glucose-6-phosphate (G6P), or 28mM glucose (Glc) supplemented growth medium. Error bars represent standard deviation between samples in three biological replicates. **=pval<0.01, ***=pval<0.001. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pif7-1*=PIF-deficient mutant in Col-0

Similar to *hxk1-3* this inhibited growth could be alleviated by G6P supplementation under both conditions, supporting our hypothesis that PIF7 regulation of hypocotyls works at least partially through G6P. In etiolated conditions, *pif7-1* is unresponsive to glucose at doses high enough to inhibit elongation in Wt and *hxk1-3*, however G6P promotes elongation in both mutants while not significantly impacting Wt. Likewise, in SD conditions G6P rescues the mutants while not significantly impacting the Wt (only a moderate reduction in length that is not significant). Interestingly, *pif7-1* does show an increased sensitivity to glucose compared to Wt, but this is not enough to compliment the phenotype. This supports our emergent hypothesis in demonstrating complementation via G6P in *pif7-1* that cannot be achieved by glucose application. These results suggest that PIF7 mediated control of hypocotyl elongation

may be dependent on modifying HXK1 activity or *HXK1* expression, consistent with previous findings in our lab's EoDFR RNA-seq experiment.

4.4 End of Day Far Red Response is Altered by G6P

After observing the previous results, our next step was to determine whether exogenous G6P supplementation could impact the start of the night response in any meaningful way and to observe how *hxk1-3* mutants act under these conditions. Based on the previous results, we can hypothesize that PIF7-mediated elongation in response to EoDFR activation will also be mediated through HXK1-G6P, and thus G6P application should restore Wt EoDFR response in a *pif7-1* mutant seedling. Our qPCR data indicates that *HXK1* expression is reduced in EoDFR conditions, we would expect *hxk1-3* mutants to show a reduced response to treatment, which we also hoped to test in this experiment. By examining seedlings grown with and without EoDFR and/or G6P, we determined that G6P presence had a significant impact on the perception of EoDFR in both *hxk1-3* and *pif7-1* seedlings, as well as altering the response in *pifQ* seedlings (Fig. 4.4).

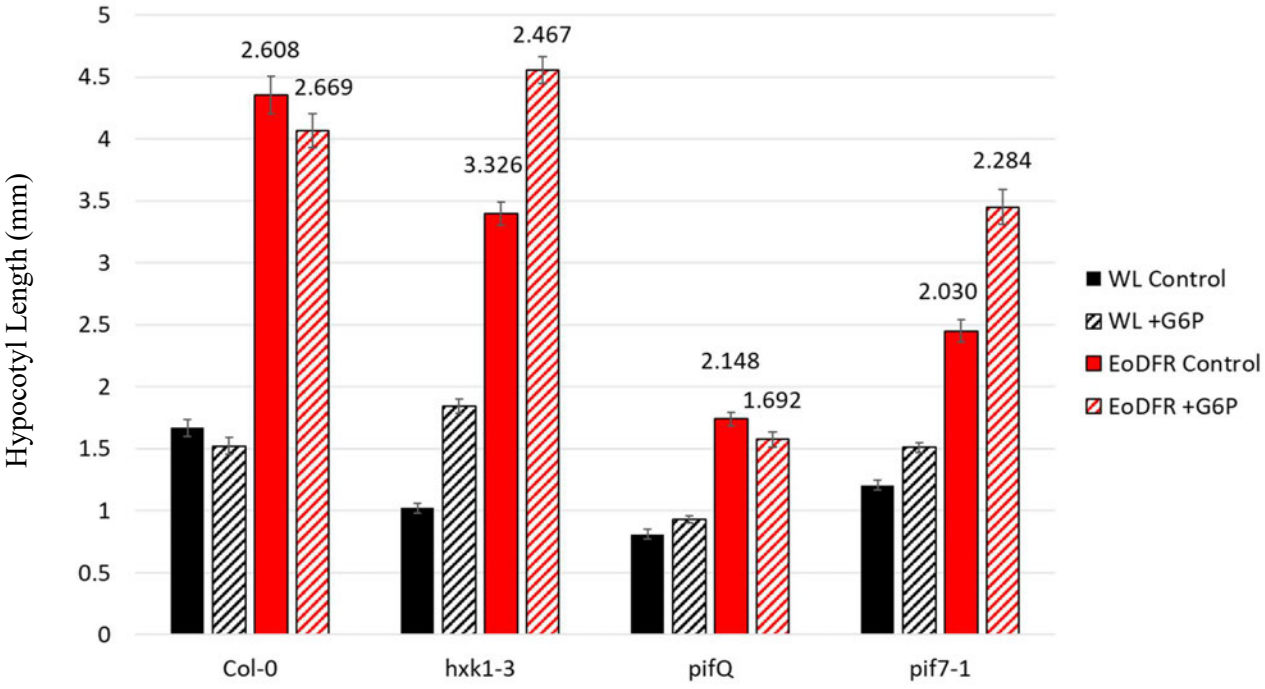

Fig. 4.4 Effects of G6P on the EoDFR Response. 4-day old seedlings grown in 8:16 standard white light (black) or with additional post-dusk FR light treatment (red) on either control (solid) or 5mM glucose-6-phosphate (G6P) (dashed) supplemented growth medium. Numbers above bars represent fold change between WL and EoDFR treated conditions. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pif7-1*=PIF-deficient mutant in Col-0. *pifQ*= PIF1, PIF3, PIF4, PIF5 quadruple mutant in Col-0

In Wt seedlings, G6P shows little to no effect on hypocotyl elongation, with only a moderate growth loss in treatment in both WL and EoDFR conditions. G6P application compliments the *hxk1-3* and *pif7*-1 phenotypes in WL conditions, and *pifQ* shows a very subtle growth response to treatment. Curiously, G6P supplementation reduced the sensitivity of *hxk1-3* and *pifQ* plants to EoDFR but enhanced the EoDFR response in *pif7-1* seedlings. A key observation in this data is that Wt is relatively blind to exogenous G6P, whereas both *pif7-1* and *hxk1-3* are responsive in both control and EoDFR conditions. Furthermore, that *pifQ* only shows marginal response to G6P, combined with the nature of *pif7-1* compared to Wt response, points to a potential link between PIF7 and HXK1. These results suggest a more complex interaction model than previously assumed, showing that G6P both enhances and inhibits growth in response to EoDFR, depending on the presence of *PIF7*, signifying a

multilevel response where G6P potentially acts in a negative feedback loop. This mechanism implies an additional unknown component acting in this pathway

4.5 Trehalose-mediated Effects on End of Day Far Red Response

Because of the documented effect of trehalose-6-phosphate (T6P) accumulation on PIF-regulated growth processes and its dependence on G6P accumulation, we investigated the possibility of T6P being a potential signaling route in the regulation of PIF7 via G6P; in this sense, we should expect to see qualitatively similar results with T6P supplementation to G6P. Because of supply issues, obtaining T6P in salt form for exogenous application was prohibitive; therefore, we began to look for alternate routes to affect this pathway. Trehalose (Tre) is metabolized from T6P through TREHALOSE 6-PHOSPHATE PHOSPHATASE (TPP). Trehalose is then metabolized into glucose via trehalase, feeding back into glycolysis; however, the application of exogenous trehalose has been demonstrated to significantly elevate internal T6P levels without affecting G6P, G1P, or F6P levels in the same manner as sucrose addition (Schluepmann et al., 2004). Other studies have demonstrated that application of 25 mM trehalose has significant negative effects on growth, particularly in root tissues (Wingler et al., 2000; Ramon et al., 2007; Garapati et al., 2015). In *Escherichia coli,* treB has been shown to directly catabolizes exogenous trehalose into T6P (Boos et al., 1990; Klein et al., 1995). While a paralog has not been described in *Arabidopsis*, trehalose supplementation could directly increase T6P levels through a mechanism that is yet to be described, as trehalose application does not have an effect on G6P accumulation (the precursor molecule of T6P) according to published work of Schluepmann et al (2004). In this context, we can infer the potential T6P network, but it should be noted that these results primarily demonstrate the effects of trehalose on this system.

To clarify the importance of trehalose in the EoDFR response, we started by growing seedlings of *hxk1-3*, *pif7-1*, and the 35S:PIF7-FLASH (PIF7-OX) overexpression line alongside Wt plants under low and moderate levels of trehalose, matching doses of G6P and glucose, as described in the previous chapter (5mM and 28mM, respectively) (Fig. 4.5).

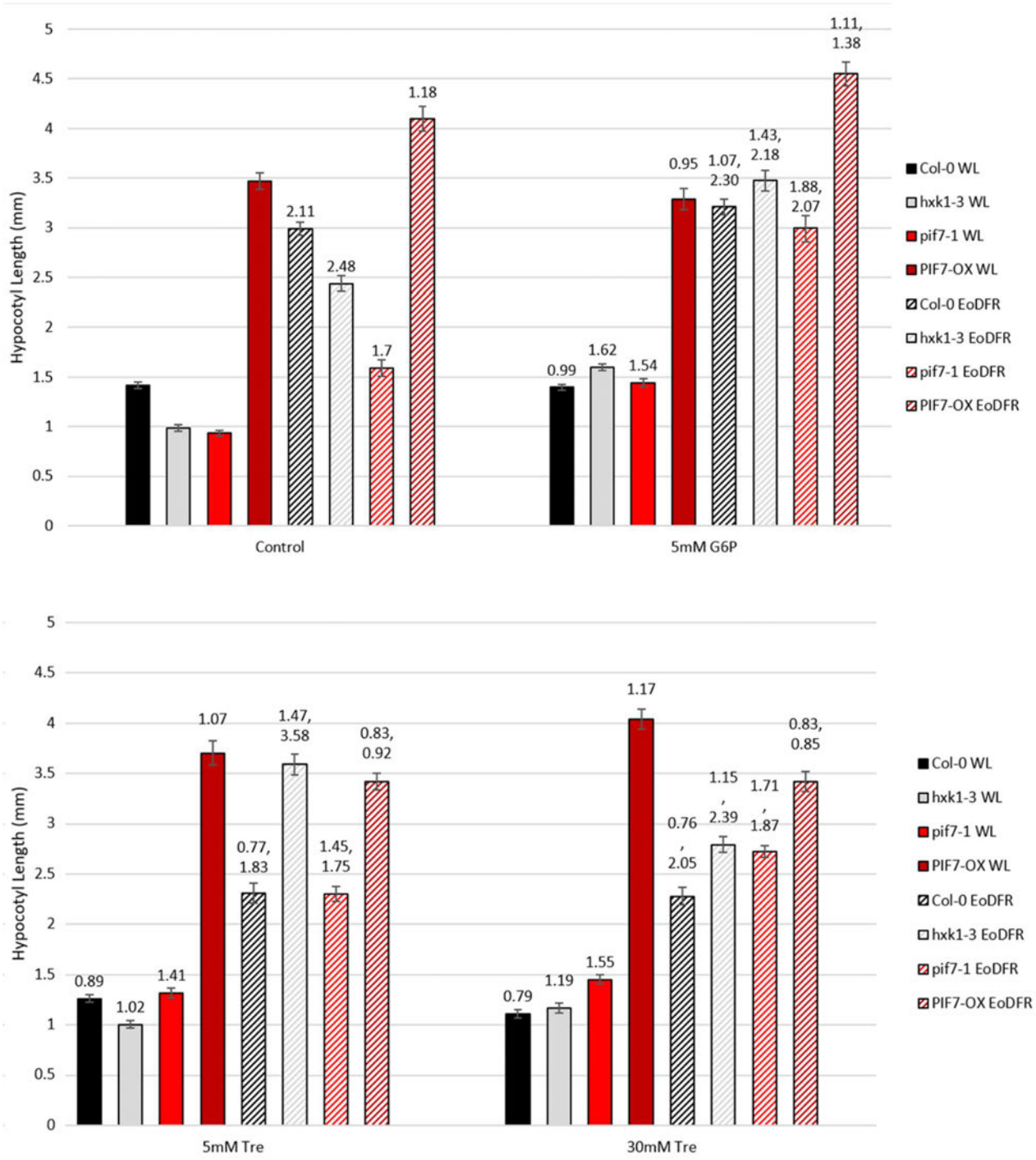

Fig 4.4. Effects of Trehalose on EoDFR Response. 4-day old Wt (black), *hxk1-3* (grey), *pif7-1* (red), and PIF7-overexpressing (PIF7-OX, dark red) seedlings grown in 8:16 standard WL (solid) and with 10 minutes of post-dusk FR treatment (dashed) on multiple types of growth medium, specified below. Numbers represent fold change from control to G6P/Tre (top) for the same light conditions, or from WL to EoDFR for the same growth medium (below). Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pif7-1*=PIF-deficient mutant in Col-0. Tre=trehalose. G6P=glucose-6-phosphate

Under white light, Wt was consistently unaffected by G6P supplementation, as was observed in this study. Consistent with previous studies, Wt seedlings showed inhibited growth with trehalose supplementation (Ramon et al, 2007). The *hxk1-3* seedlings showed no response to exogenous trehalose at lower doses and exhibited a slight growth boost at higher doses. *pif7-1* seedlings demonstrated strong promotion of growth with increasing doses of trehalose, while PIF7 overexpressing lines show subtle growth increases only at higher dosages. However, we observed a shift in these responses under the EoDFR conditions. Col-0 still showed inhibition, but when PIF7 was activated, we observed a similar reduction in growth in the PIF7 overexpressing lines, and *hxk1-3* seedlings responded to trehalose at low dosages, identical to that of *pif7-1.* This suggests that trehalose perception is important for growth inhibition by repressing PIF7 when it is active, although it also shows that trehalose can promote growth in a PIF7-independent manner. This experiment also shows that the EoDFR response is enhanced by G6P in the PIF7-OX lines.

When looking at the effect of trehalose on the EoDFR response, we can see that while it doesn't strongly impact the Wt response, only a lower dose amplifies the response in *hxk1-3.* These results suggest an interaction between trehalose/T6P and PIF7, which is unique compared to the published work on PIF4 in its temperature-dependent signalling role where T6P-mediated inactivation of KIN10 results in the activation of PIF4-dependent growth (Hwang et al., 2019). To clarify this interaction site, we sought to understand the effect of G6P and trehalose supplementation on other PIF family members, particularly PIF4 and PIF5.

4.6 Effect of G6P and Trehalose on Various *pif* Single and Multi-allele Mutants

Previously, we observed that G6P did not significantly affect the growth of *the pifQ* quadruple mutant seedlings (Fig. 3.9); however, given the significance of the *pif7-1* response, we deemed it necessary to reinvestigate the single mutants for *PIF4* and *PIF5*, as well as their double and triple (with *PIF7*) mutant alleles, because of their published importance in hypocotyl growth (Kunihiro et al, 2010; Kunihiro et al, 2011; Casal, 2012; Lan et al, 2022). With this in mind, we can theorize that the control of hypocotyl through the *PIF4-PIF5-PIF7* nexus is influenced by carbon reserve status through G6P/Tre accumulation, and as such we would expect to see perturbations in the response to these metabolites in the mutant alleles. We examined the responses of these mutant lines to EoDFR and WL conditions under control, G6P, and trehalose treatment (Fig. 4.6).

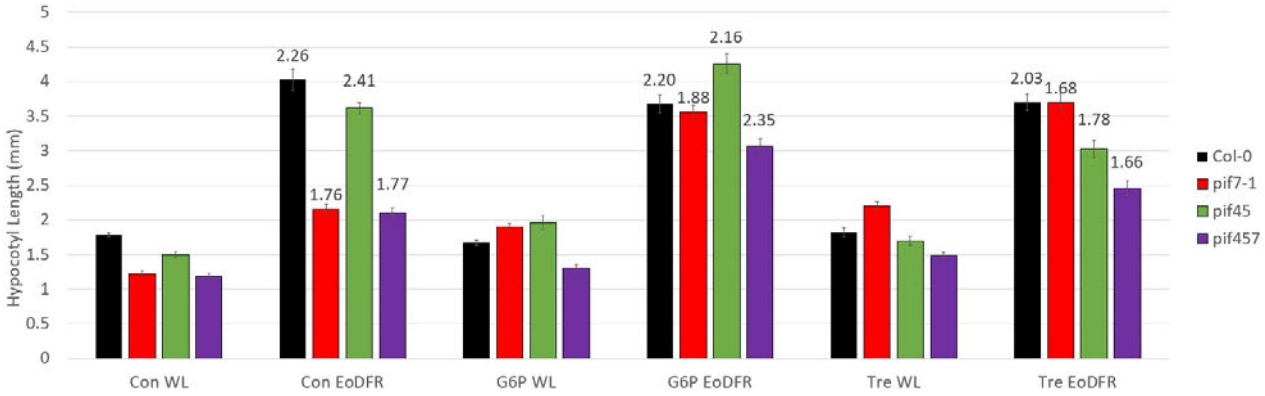

Fig. 4.6 Effects of G6P and Trehalose on EoDFR Response in Other *pif* Mutants. 4-day old Wt (black), *pif7-1* (red), *pif45* (green), and *pif457* (purple) seedlings grown in 8:16 standard WL (solid) and with 10 minutes of post-dusk FR treatment (dashed) on multiple types of growth medium, specified below bars; control, 5mM G6P, or 5mM Tre. Numbers above bars represent fold change from WL to EoDFR. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type). *pif#*=PIF#-deficient mutant in Col-0

Under WL conditions, the G6P-mediated rescue of *pif7-1* was consistent with previous findings, *pif45* short hypocotyl was rescued by G6P supplementation (akin to the *pif7-1* and *hxk1-3* phenotypes) although the triple mutant *pif457* did not significantly elongate. This suggests that under these conditions, G6P mediated growth requires the presence of PIF4 and PIF5 to respond to treatment in a Wt manner indicating that PIF7 inhibits the G6P-mediated hypocotyl elongation, and this action is opposed by PIF4 and PIF5.

In EoDFR, we observed a largely similar suite of responses to G6P treatment in all cases, except those where *PIF5* was absent. The double *pif45* mutant responded to G6P treatment, thereby rescuing its short hypocotyl. The *pif457* triple mutant seedlings responded to the treatment and showed a 1.41-fold increase in length, although this growth rate was insufficient to rescue the short hypocotyl phenotype. This suggests that PIF4-5 play a significant role in the evening shade avoidance response to FR light, which is consistent with previous work on the shade avoidance response (Mizuno et al., 2015). In response to the addition of trehalose, we observed a slight increase in the length of *pif457* seedlings. However, the growth of the *pif45* double mutant was significantly inhibited by trehalose supplementation. This suggests that PIF7 mediated growth is blocked by trehalose supplementation while also stimulating other growth methods. This is consistent with our previous findings and further supports our hypothesis that trehalose/T6P reacts uniquely with PIF7 compared to the published understanding of how it regulates PIF5. To further test this theory, we observed the response of these lines, as well as the single *pif4* and *pif5* mutants to increasing doses of trehalose, G6P, and glucose in order to test both the specificity of the trehalose response (i.e., it does not simply add more glucose into the system through its breakdown via trehalase) and to see how it affects the system dynamically under both WL and EoDFR conditions (Fig. 4.7).

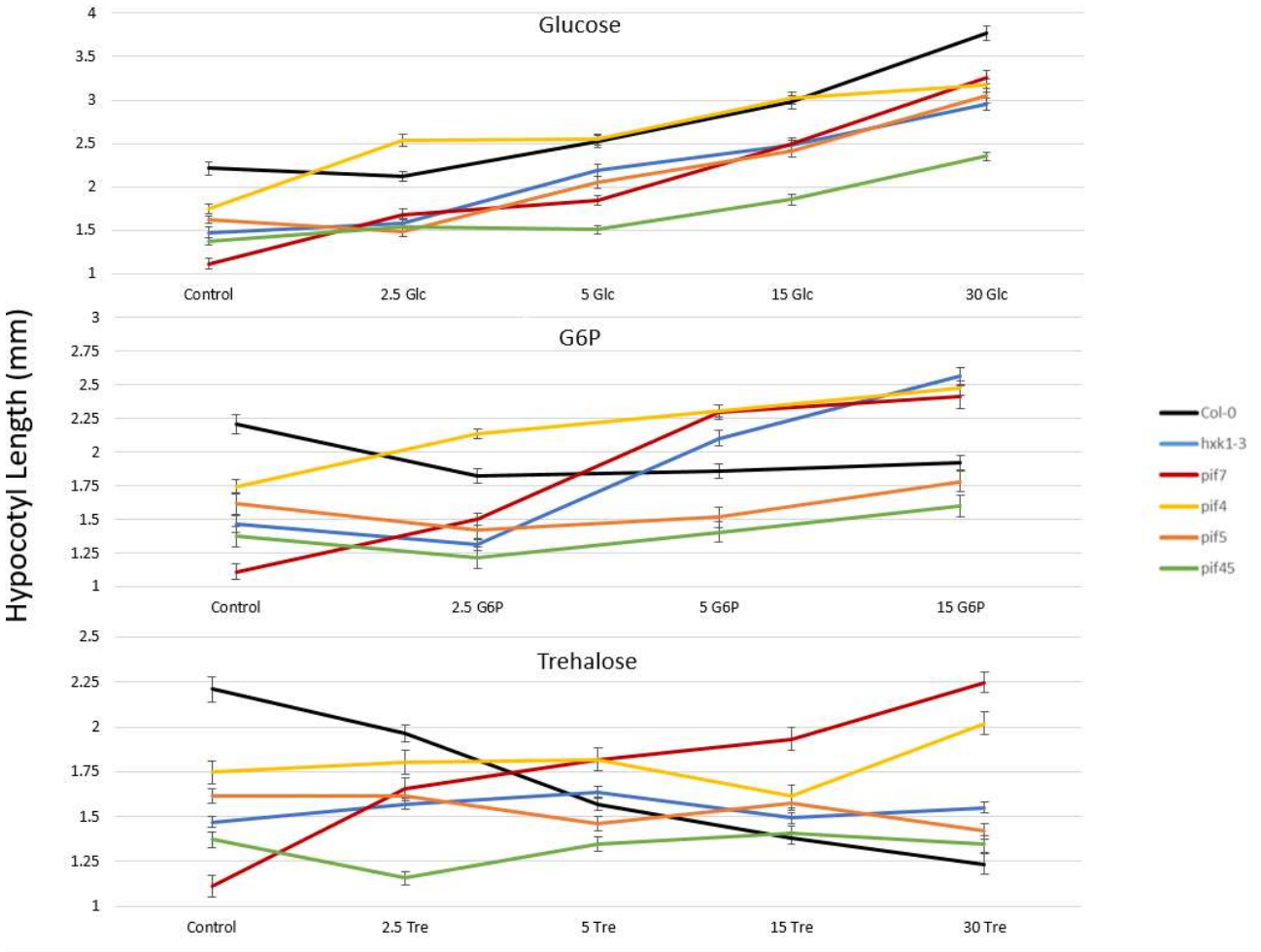

Fig. 4.7 Dose-Response Curves for Glucose, G6P, and Trehalose on Various *pif* Mutants. 4-day old Wt (black), *hxk1-3* (blue), *pif7-1* (red), *pif4* (yellow), *pif5* (orange), and *pif45* (green) seedlings grown in 8:16 standard WL on increasing concentrations of glucose (Glc) (upper), G6P (middle), or trehalose (Tre) (lower) supplemented growth medium. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *hxk1-3*=HXK1-deficient mutant in Col-0. *pif*#=PIF#-deficient mutant in Col-0

Under white light, we observed a consistent lack of response to Tre for *pif4* and *pif45* from the lowest to the highest dose. *hxk1-3* also did not respond to Tre treatment, which is consistent with the previous observations and suggests that it may be required for the Tre hypocotyl response. Wt and *pif7*-1 plants responded significantly to Tre treatment in opposite directions: *pif7-1* showed a gradual increase in length, akin to its response to glucose and G6P (as previously observed) but Col-0 plants continuously diminished in length throughout the curve. This suggests that trehalose is uniquely perceived by plants and is not simply catabolized into glucose molecules. Furthermore, this demonstrates that PIF7 has significant

control over the response to trehalose under these conditions, although the other observed PIFs and HXK1 exhibited an altered response to Wt. This is consistent with the proposed interaction our previous data would indicate.

Combined with our observations on the response of the *pif457* triple mutant to Tre application, it seems that the method by which PIF7 responds to trehalose is hypostatic to the PIF4/5 response mode, suggesting that PIF7 suppresses growth in response to Tre functions through the regulation of PIF4 and 5. This hypothesis is consistent with published theory that phytochrome-dependent PIF7 regulation works through modifications of PIF4 and 5 expression and potential protein-protein interactions (Leivar et al, 2020). To summarize what the data shows us:

- Glucose increases hypocotyl elongation in all mutants and Wt, but *pif7-1* shows an enhanced sensitivity to glucose, and *pif45* has a reduced response
- G6P is mildly inhibitory to growth in Wt but promotes growth in both *pif7-1* and *hxk1-3. Pif5* and *pif45* are unresponsive to G6P treatment, and *pif4* shows a slight response.
- Trehalose is inhibitory to Wt and promotes growth in *pif7-1*, while all other genotypes are largely insensitive to treatment.

Given the similarities in response to G6P, we can hypothesize that PIF7 regulation of the hypocotyl must work at least partly through HXK1-G6P-Tre signaling. To test this hypothesis, we started a genetic cross between the two single mutant alleles for *hxk1-3* and *pif7-1*. Unfortunately, we were unable to analyse their response to EoDFR and WL for this study.

However, we were able to mimic the effects of *hxk1-3* mutation in Wt in chapter 2 of this thesis using specific inhibitor molecules. So, we can preliminarily test our hypothesis using *pif7-1* seedlings grown in the presence of HXK1 inhibitor molecule 2-deoxy-D-glucose (2-DG) under WL and EoDFR conditions in short-day photoperiodic conditions (Fig. 4.8).

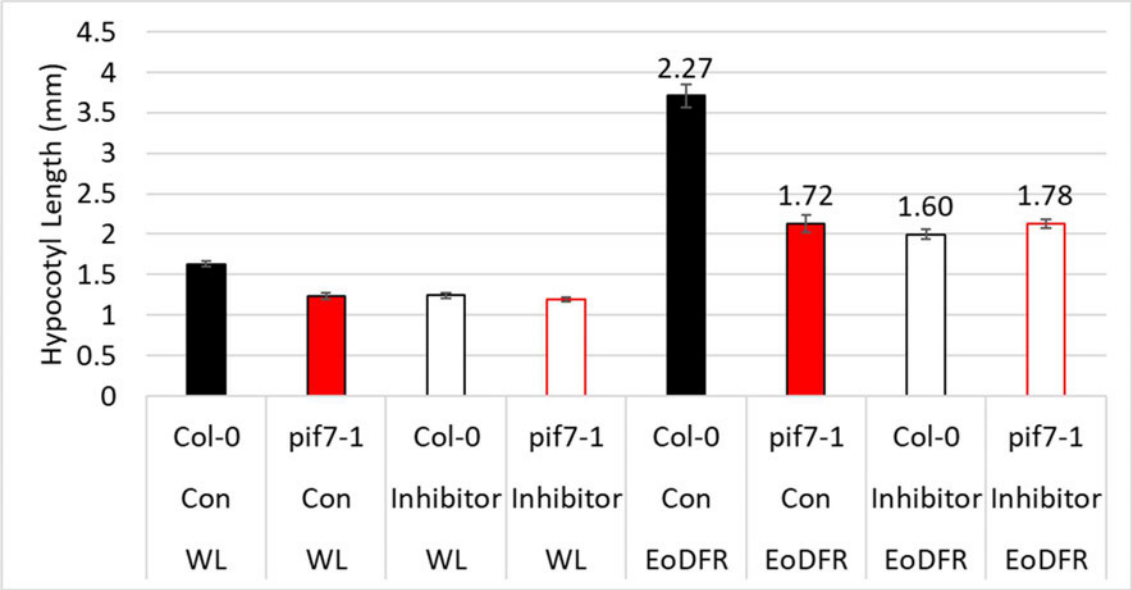

Fig. 4.8 Effect of HXK Inhibitor 2-DG on EoDFR in *pif7-1* and Wt. 4-day old Wt (black) and *pif7-1* (red) seedlings grown in 8:16 standard WL (left) or with 10 minutes of post-dusk FR treatment (right) in control (solid) or 0.05mM 2-DG (clear) supplemented growth medium. Numbers above bars represent fold change between WL and EoDFR treatments for the same type of medium and genotype. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *pif7*=PIF7-deficient mutant in Col-0. Inhibitor=2-DG=2-deoxyglucose

In this experiment, as previously demonstrated in WL, Wt seedlings are significantly shorter when 2-DG is present in the growth medium, and we also see that is maintained under EoDFR conditions. Interestingly, we show that the EoDFR response is significantly reduced in treatment, going from a 2.28x fold-change with no inhibitor to a 1.6x fold-change with 2-DG. *pif7-1* seedlings are unresponsive to treatment in WL and in EoDFR, with no change in response to EoDFR treatment in the presence of inhibitor. This data supports our emergent hypothesis that the EoDFR response through PIF7 requires HXK1, and as such PIF7 moderates hypocotyl elongation through HXK1 regulation.

4.7 *tps1* Mutants show Altered End of Day Response

To understand the implied role of trehalose synthesis in the EoDFR response, we procured the *tps1-12* mutant analysed in Gómez et al. (2010), a non-embryo lethal allele demonstrated to have significantly diminished levels of T6P (approximately halved), creating an environment suitable for understanding the impact of T6P on the EoDFR response. As such, we grew these seedlings next to Wt and *pif7-1* seedlings to observe their response to EoDFR treatment, and we observed that *tps1-12* seedlings show a significantly diminished response to EoDFR to the same degree as *pif7-1* seedlings, with a 2.1x change in both mutant seeds compared to a 2.7x change in the Wt (Fig. 4.9).

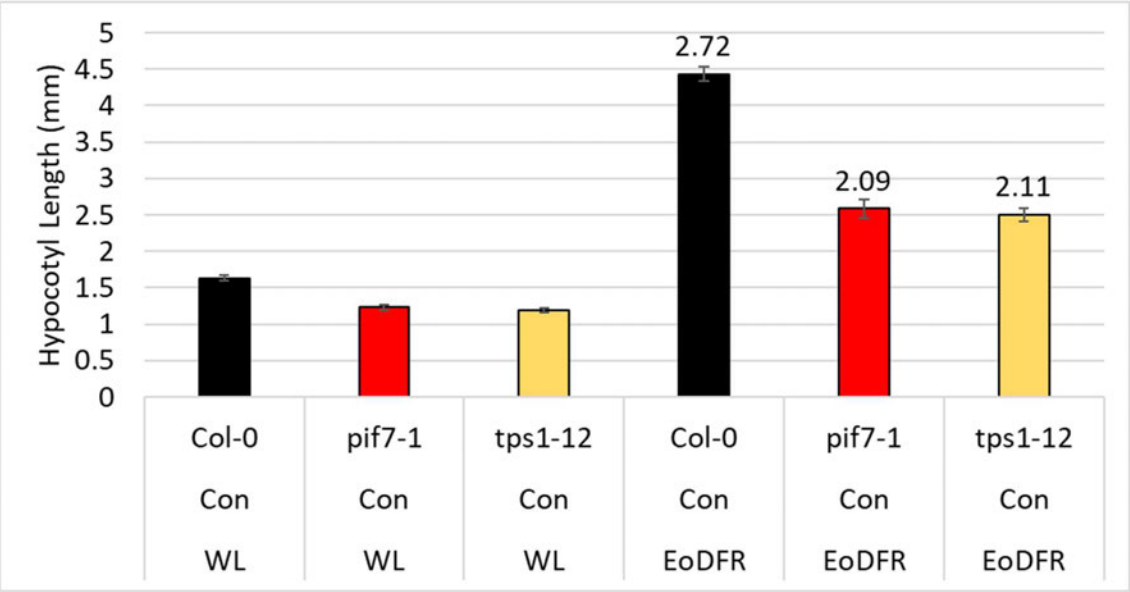

Fig. 4.9 Effect of Trehalose Synthesis Gene on EoDFR Response. 4-day old Wt (black), *pif7-1* (red), and *tps1-12* (yellow) seedlings grown in 8:16 standard WL (left) or with 10 minutes of post-dusk FR treatment (right). Numbers above bars represent fold change between WL and EoDFR treatments for the same genotype. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *pif7*=PIF7-deficient mutant in Col-0. *tps1-12*= TPS1 mutant in Col-0

These results demonstrate that under WL conditions, PIF7-mediated growth occurs primarily through HXK1 activity, as inhibition has no impact on the growth of these seedlings. However, there seems to be some separation between PIF7 and HXK in regulating growth, as the inhibitor significantly decreased the hypocotyl length of *the pif7-1* mutants. The TPS/T6P pathway seems to be implicated in this response, as *tps1-12* mutants did not respond to inhibitors in either treatment. To further examine the effects of this system on the EoDFR

response, we analysed the growth of inducible SnRK RNAi mutant lines from Baena-González et al (2007) and their response to EoDFR treatment (Fig. 4.10)

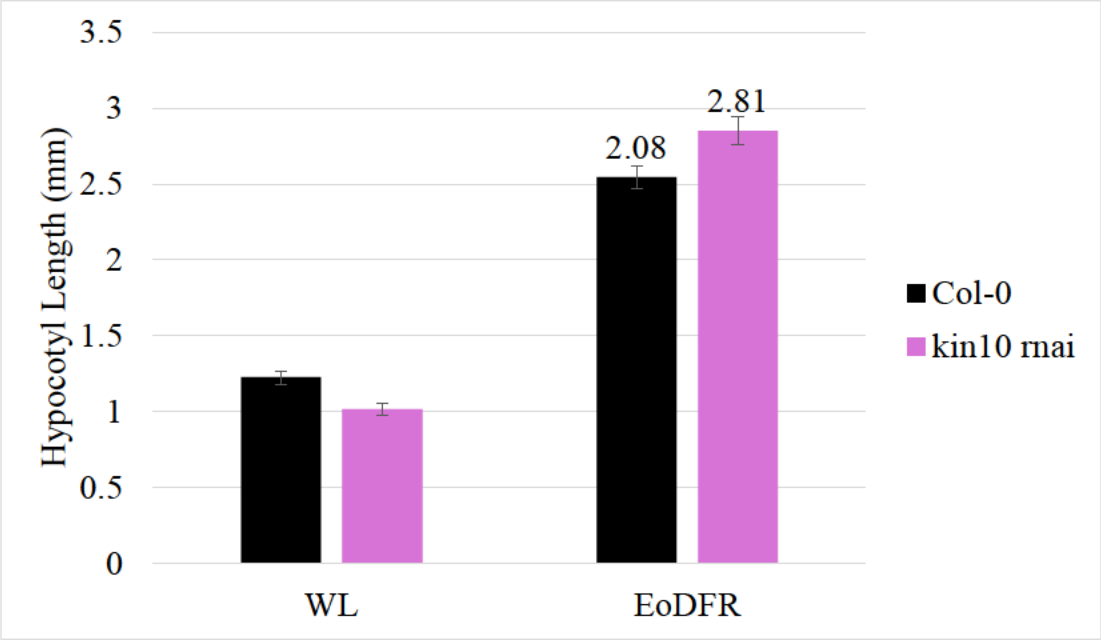

Fig. 4.10 SnRK1 Inhibition Enhances EoDFR Response. 4-day old Wt (black) and *kin10-RNAi* (pink) seedlings grown in 8:16 standard WL (left) or with 10 minutes of post-dusk FR treatment (right). Numbers above bars represent fold change between WL and EoDFR treatments for the same genotype. Error bars represent standard deviation between samples in three biological replicates. Col-0=Columbia (wild-type) *kin10rnai*=Inducible KIN10 mutant line in Col-0

In this experiment, Wt responds to EoDFR treatment as expected, but the *kin10* RNAi line shows a significantly enhanced response to EoDFR, with an increased fold change of 2.81x compared to the 2.08x fold change in Wt. These results coincide with our expectations from the previous experiment, which would lead us to expect an enhanced response to EoDFR in the mutant lines, further strengthening our emerging hypothesis that T6P-SnRK1 activity is involved in suppressing the PIF7-dependent elongation response

4.10 Discussion

Published works have underlined the importance of PIF7 and the shade-avoidance response in the development of seedlings, highlighting its distinct regulatory capacity over this response (Leivar et al, 2008, Huang et al, 2018). Our research supports these conclusions by re-creating results that demonstrate the importance of PIF7 not only in EoDFR conditions, but also in light-limiting conditions. Furthermore, we discovered that unlike other *PIF* mutants, *pif7-1* mutant hypocotyls respond to G6P treatment, significantly elongating to rescue a Wt growth mode. This suggests a unique mode of growth regulation via PIF7 that works through moderating glucose phosphorylation via HXK1 phosphorylation or downstream processes, in which PIF7 promotion of *HXK1* results in successful mobilisation of seed reserves and activation of growth as described in detail in chapter 2.

Following this work, we wanted to further clarify the relationship between *PIF7* and *HXK* by looking at the regulation of these genes. *PIF7* transcriptomic analysis in Burko et al (2022) reveals that *HXK1* expression is regulated by PIF7 in thermomorphogenesis, and our qPCR results demonstrate that this is conserved in the EoDFR response as well, showing significantly reduced *HXK1* expression in *pif7-1* when in Wt FR light induces high expression of *HXK1*. Other *HXK* family genes, however, do not show significant misregulation via *pif7-1* under FR enriched conditions, suggesting that the other members of the family are regulated in a different capacity to *HXK1* in this response. This, and the opposite direction of regulation compared to the RNA-seq data could potentially be explained by opposing patterns of regulation based on life stage and/or tissue type; previous work in the lab that showed EoDFR-dependent misregulation was done in adult leaf tissue whereas this experiment was performed in seedlings. Different tissue types have an understood change in the direction of regulation for genes; for instance, far-red light excitation results in petiole elongation contrasted to laminal growth reduction as a result of the identity of the tissue type affected by the same signal (de Wit et al, 2015). It is also possible that PIF8, another PIF gene implicated in shade avoidance, could potentially be responsible for their regulation as FR light still induces expression of these genes.

While this all seemed straightforward, we had noticed that our data could not be explained by a simple A → B → C diagram, and that there was another piece of this puzzle that we had not yet identified. (Fig. 4.11)

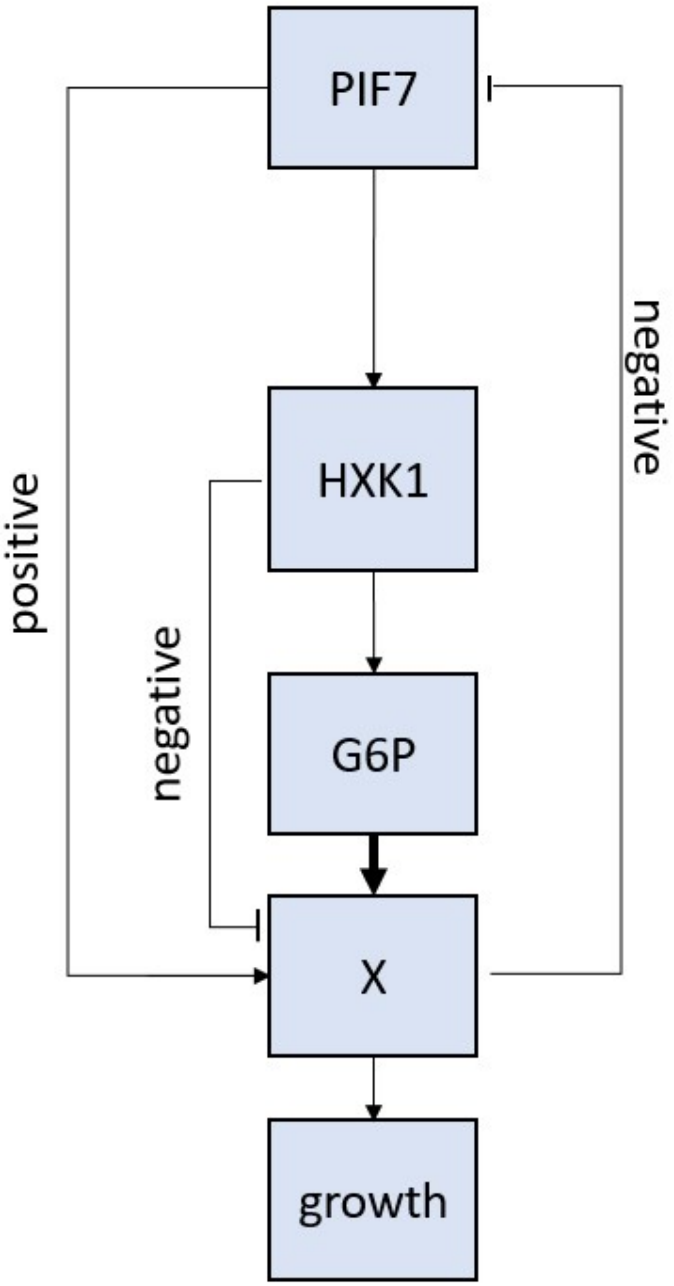

Fig. 4.11 Hypothetical Model of HXK and PIF7 Control of Hypocotyl Elongation. This early model suggested a linear correlation between PIF7 activity and growth with an undescribed effector "x" that reciprocally inhibited PIF7 activity, as G6P activity seems to both enhance and inhibit PIF7. G6P=glucose-6-phosphate. →=activation −| =repression

When searching for similar mechanisms of metabolism and light intersection, we noticed an abundance of work being done on the relationship between PIFs and SnRK1 and highlighting the importance of PIFs in sucrose-induced elongation (Liu et al, 2011, Stewart et al, 2011,

Simon et al, 2017, Kelly et al, 2021). From evaluating the effects of trehalose on the growth of *pif* mutants, we demonstrated that trehalose supplementation reduces the length of Wt hypocotyls, has no effect of *hxk1-3, pif4, pif5,* and *pif45* hypocotyls, but promotes growth in *pif7-1*. T6P has been understood to promote elongation through stabilisation of PIF4 in thermogenic response by destabilising SnRK1, with *tps* mutant seedlings being short in 28°C when grown in continuous light but indistinguishable from Wt at 20°C (Hwang et al, 2019). In this context, it would initially seem that our *pif4* results are inconsistent with the literature, where PIF4 activity is reported to be promoted by the inhibition of SnRK1 via T6P accumulation, but it is important to recall that this experiment utilized trehalose rather than T6P; our *pif45* data however is consistent with these expectations. Furthermore, our work with the *pif457* triple mutant demonstrates that the combined presence of PIF4 and PIF5 are epistatic to PIF7 in hypocotyl growth

Incidentally, it appears that trehalose/T6P regulation of growth may work differently when in light limiting conditions, as our results in short day photoperiod growth show that *tps1* mutants are short in 20°C. Furthermore, our *pif7-1* dose-response curve demonstrates that PIF7 regulation of elongation in WL conditions likely works through a G6P/Tre-dependent signaling pathway, and *hxk1-3* seems to be required for this response given its lack of sensitivity to trehalose supplementation. A key follow-up experiment would be to analyse the response of the *hxk1-3*x*pif7-1* double mutant line, which was generated for this project but not analysed in this thesis due to time constraints. However, our preliminary data using the HXK inhibitor molecule 2-deoxy-D-glucose does support this hypothesis, given that HXK inhibitor significantly impacts the EoDFR response in Wt but not for *pif7-1* seedlings. Additionally, as mentioned previously a recent RNAseq/ChIPseq dataset demonstrates that PIF7 binds to the *HXK1* promoter *in vivo* as well, providing further support for this emergent hypothesis and indicating that PIF7 regulation of *HXK1* is pre-transcriptional. (Burko et al, 2022). An analysis of the predicted promoter regions of all *HXK* family genes shows G/E boxes that PIF7 canonically binds to (Fig. 4.1b).

Given that *tps1-12* mutants demonstrate a diminished response to EoDFR treatment, and a SnRK1-impaired *kin10-RNAi* line results in an enhanced EoDFR response, we can then speculate that PIF7 and SnRK1 may interact in a different manner than that of other PIFs in WL. In EoDFR conditions they interact similarly to PIF4/SnRK. Due to PIF7's unique characteristics, such as its continued presence in the cytosol when phosphorylated and it's poly-Q domain (Freiman and Tijan, 2002, Huang et al., 2018), these properties may allow a

different relationship between it and SnRK depending on the environmental signals in play and its location in the cell. The RNAseq from Burko et al (2022) also shows significant misregulation of multiple TPS genes in EoDFR conditions, which could suggest that these SnRK1 regulated genes may be useful as a transcriptional target readout of SnRK1 activity in further studies.

4.11 Conclusion

In this chapter, we delved into the intricate relationship between *PIF7*, *HXK1*, and various sugar molecules, shedding light on the mechanisms underlying plant growth responses to light and sugars. First, we highlighted the unique characteristics of *PIF7*, a key player in regulating responses to far-red light at the end of the day. Unlike other PIF family members, PIF7 possesses distinct structural features and exhibits non-standard behaviour when phosphorylated, remaining in the cytosol through interaction with 14-3-3 proteins.

We then explored the relationship between PIF7 and HXK1, utilizing RNA-seq data to demonstrate significant misregulation of *HXK* family genes in response to far-red light treatment. Notably, *pif7-1* mutants displayed altered hypocotyl growth under different light conditions, which could be modulated by the addition of G6P. These findings suggested a potential link between PIF7 and HXK1 activity. Furthermore, we investigated how exogenous G6P supplementation influenced the EoDFR response, revealing complex interactions between sugars and PIFs. G6P's presence significantly impacted the perception of EoDFR, with varying effects on different mutants, indicating a complex regulatory network.

We examined various *pif* mutants' responses to G6P and trehalose under different light conditions, revealing specific requirements for PIF4, PIF5, and PIF7 in mediating sugar-induced growth responses. These findings expanded our understanding of the intricate interplay between sugar signaling and PIFs. Our study also demonstrated that PIF7 positively regulates *HXK1* expression in EoDFR conditions, further implicating its role in this intricate signaling pathway. We confirmed this regulation through qPCR analysis and found that PIF7's impact on *HXK1* expression was most pronounced in EoDFR conditions.

To explore the role of trehalose in this system, we utilized trehalose supplementation to modulate T6P levels indirectly. Trehalose's effects on growth varied across different mutants, suggesting a unique interaction between trehalose/T6P and PIF7, distinct from previously

known interactions with PIF4. Finally, we investigated the role of trehalose synthesis by analysing *tps1-12* mutants. These mutants displayed altered responses to EoDFR treatment, akin to *pif7-1* mutants, emphasizing the importance of T6P-SnRK1 in regulating the EoDFR response.

In conclusion, our research uncovers a highly complex and interconnected network involving PIF7, HXK1, and sugar molecules in the regulation of plant growth responses to light conditions. These findings deepen our understanding of plant signaling pathways and provide a foundation for further exploration of these intricate interactions. (Fig. 4.12)

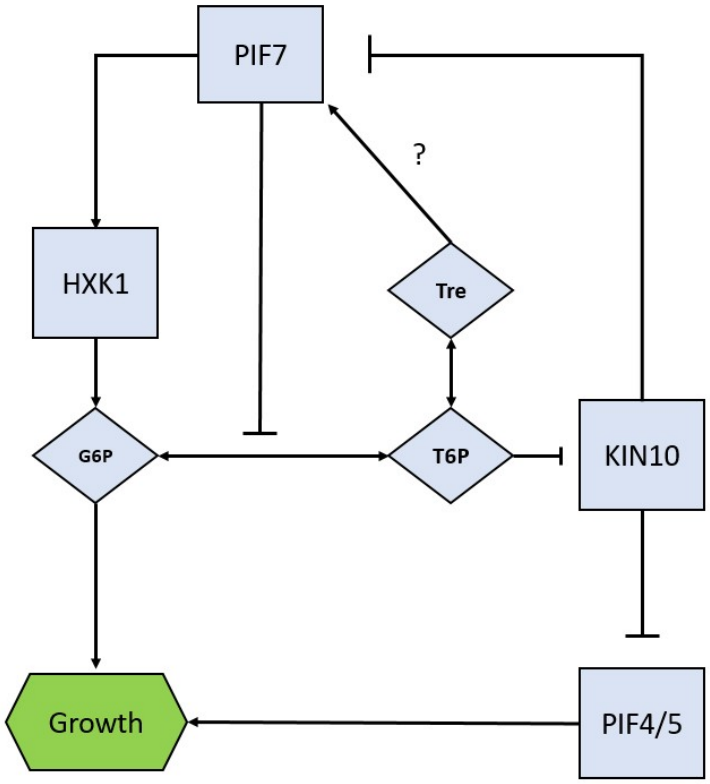

Fig. 4.12 Updated Hypothetical Model of HXK1, SnRK1, and PIF Control of Hypocotyl Elongation. This model proposes a cyclical network of regulation between HXK1, SnRK1, and PIF7. HXK1-G6P-T6P is known to inhibit KIN10 and can be seen in our data, and our data also suggests that PIF7 via shade can influence the accumulation of these metabolites and thus affect KIN10 as well. KIN10 activity seems to repress the PIF7-EoDFR response based on preliminary results. PIF7 may activate KIN10 based on PIF7-Ox attenuating the G6P response. G6P=glucose-6-phosphate. T6P=trehalose-6-phosphate. Tre=trehalose. →=activation −| =repression

To further test this model, it may be pertinent to examine more specific and simple phenotypes for each component affected in the model; for instance, using putative TPS gene

expression as a readout of KIN10 activity, or by quantifying levels of metabolites such as glucose and G6P in these mutants as well.

Chapter 5. Conclusions

Plant growth regulation is a complex network orchestrated by intricate molecular players, and our investigation has delved into the multifaceted role of HEXOKINASE 1 (HXK1) in this intricate system. By examining the genetic and molecular underpinnings of the *gin2-1* mutant, we have explored not only the regulatory networks governing plant growth but also the interconnected relationships between HXK1, SnRK1, PIF signaling, and blue light perception. This study ties together three distinct perspectives, highlighting the complexity and nuances that govern plant growth responses to light, sugars, and enzymatic processes.

In this study, we established the importance of HXK1's enzymatic role in seedling growth through various approaches. By examining *hxk1-3* mutant seedlings' response to G6P supplementation and glycolytic inhibitor molecules, we demonstrated the critical role of G6P synthesis in hypocotyl development. Replicating published results in key HXK1 papers affirmed the validity of our experimental methods and growth conditions. The conclusion drawn is that HXK1 signaling drives growth once photosynthesis is activated and internal sugar levels are elevated, suggesting its role in catabolizing seed reserves for growth.

Our exploration into the *gin2-1* mutant revealed a significant misregulation of 2344 genes, showcasing distinct patterns of upregulation and downregulation. The upregulated genes pointed towards a metabolic shift, emphasizing HXK1's role beyond its canonical metabolic functions. qPCR validation shed light on discrepancies observed in the RNA-seq data, calling for a deeper understanding of the observed regulation patterns. The intersection of HXK1 and SnRK1 revealed a coordinated response to metabolic signals, yet the unique misregulation in *gin2-1*, especially in genes associated with cell cycle regulation and plastid genes, unveiled a novel role for HXK1 in chloroplast transcriptome regulation.

Exploration into PIF signaling illuminated intriguing connections between light signaling pathways and HXK1-mediated growth. The *pifQ* mutant's response paralleled the phenotypic characteristics of *gin2-1*, hinting at potential crosstalk between these pathways. However, the distinct responses of *pifQ* to glucose and G6P supplementation provided interesting results

requiring further investigation. Our comprehensive exploration into the genetic and molecular intricacies of the *gin2-1* mutant transcriptome, and examinations of genes regulated between HXK1, SnRK1, and PIF signaling paints a vivid picture of the complexity underlying plant growth regulation, demonstrating distinction in the ways that HXK1 regulates growth both through G6P and potentially independently of its G6P production. The orchestra of molecular interactions involving HXK1 begins to unfold, and future research endeavours should delve deeper into the molecular mechanisms governing these interactions providing valuable insights for crop improvement and addressing challenges in agricultural sustainability.

A dedicated exploration into the interactions between PIF7 and HXK1 uncovered the unique characteristics of PIF7 in far-red light responses. Significant misregulation of *HXK* family genes in response to far-red light treatment suggested a potential link between PIF7 and HXK1 activity. Exogenous G6P supplementation influenced the EoDFR response, revealing intricate interactions between sugar molecules and PIF7. Various pif mutants' responses to G6P and trehalose under different light conditions expanded our understanding by demonstrating unique responses to increasing amounts of soluble sugars. Trehalose supplementation demonstrated unique interactions between trehalose/T6P and PIF7, distinct from known interactions with PIF4.

Following this study, the most important step in expanding on the hypotheses laid out in this thesis is the generation of crossbreeds between key mutant lines in order to fully understand the order and coordination of these regulatory pathways through these important factors. These lines will be essential to fully understand the ways in which HXK1 controls plant development. Furthermore, work should be done on adult plants, where HXK1 has been shown to have a significant role in controlling leaf development and expansion (van Dingenen et al, 2018). Understanding how these pathways operate in both seedling and adult life stages will allow for much more depth when expanding the findings of this study beyond the lab and into potentially agricultural developments. For instance, elaborating on how metabolite concentration influences the response to neighbour detection via shade (far-red light detection) could result in unique supplements being added to allow for a higher density of crops in a field, or in canopy shaded crops such as peanuts allow for potentially more robust yields through similar methods.

Chapter 6. Methods

Plant material, growth conditions, and treatments.

The wild-type *Arabidopsis thaliana* ecotypes used in this study are Landsberg *erecta* (Ler) and Columbia-0 (Col). The seeds for mutants *gin2-1* (Ler), *hxk1-3* (Col), *pfk1-1* (Col), *vha-b1* (Col), *rpt5b* (Col), *king1* (Col), *tps1-12* (Col-0), *kin10rnai* were obtained from The Nottingham *Arabidopsis* Stock Centre (NASC), UK. *hxk1*^{S177A} seeds were kindly shared by Professor Ruth Stadler. For all experiments, seeds were surface-sterilized for 10 minutes with a mixture of 30% thin bleach and 0.005% Triton X-100 in water while vortexing. Seedlings were then washed with sterile water, suspended in 0.1% agar solution, and stratified in darkness for 2-3 days at 4°C. Afterwards, all seeds were sown on 0.5X MS plates (0.8% agar, pH 5.7) and grown in 20°C in 8:16 photoperiodic white light of 80 µmol $m^{-2}s^{-1}$ unless otherwise specified. For glucose-6-phosphate (G6P) (Sigma G7879), sodium pyruvate (Sigma P2256), mannoheptose (Sigma M6909), N-acetyl-D-glucosamine (SLS A8625-5G), trehalose (B7916-APE), 2-deoxy-D-glucose (B1027-APE-5g), and glucose (Sigma G8270) treatments, desired concentrations from filter sterilized stocks were added to sterilized media and seeds directly sown. Unless otherwise stated, all experiments were performed in triplicate with 40 seedlings per replicate.

Seedling hypocotyl length measurements.

Images of seedlings laid flat on growth media was used to quantify hypocotyl length and cotyledon area using ImageJ (NIH, Maryland). A ruler was placed alongside the plated seedlings to add scale in the analysis, taken by the average of five measurements to standardise 1 mm. Seedlings were traced using the software, then measured for length in pixels, converted to millimetre measurements using the aforementioned standard. Hypocotyls were selected for measurement based on similar germination instances to ensure germination differences were not included in the data, and that only differences in post-germinative growth were considered.

Seedling hypocotyl cell counts and length measurements.

For determination of etiolated hypocotyl epidermal cell lengths/numbers, seedlings were mounted on slides and visualized using Eclipse E600 Nikon DIC microscope. Individual cell lengths from each section of the hypocotyl (basal, middle, upper) were defined and standardised based on their relation to the root and shoot apical meristems, and then

measured in pixels using ImageJ (standardised to 1 micrometre using a standardisation slide). Cell number per file was obtained by manual count under the microscope. Unless otherwise stated, all experiments were performed in triplicate with at least 20 seedlings per replicate.

Glucose quantification.

Seedlings were harvested in liquid nitrogen, finely ground and ethanol extracted thrice. Glucose was then quantified using an enzymatic assay at 340nm wavelength in a spectrophotometer; at 340 nm, we can detect approximate levels of NAD in the extract. HXK enzyme was then added to the extract, and the discrepancy between the values was put into the following equation to determine the amount of glucose in the extract:

$$\Delta A \; x \; \frac{V_1 \; x \; 180.16}{6.3 \; x \; 0.55 \; x \; V_2 \; x \; 1000}$$

Where ΔA is the difference in absorption by NAD between pre-enzyme and post-enzyme stabilisation points, V_1 is the volume of the sample, V_2 is the volume of ethanol extract added to the mixture, 180.16 is the molecular weight of glucose, and the other values are constants. Unless otherwise stated, all experiments were performed in triplicate with 50 seedlings per replicate.

Chlorophyll measurements

Seedlings were weighed in samples ranging from 50-100 mg, harvested into liquid nitrogen, pulverised into a fine powder, then acetone extracted three times. Ethanol extract was used to quantify absorption at 663, 652, 646, and 710 nm and standardised to a blank of acetone in a spectrophotometer. Quantity of chlorophyll A was calculated with the following equation:

$$12.21(\Delta A \; 663 - 710) - 2.81(\Delta A \; 646 - 710)$$

Quantity of chlorophyll B was calculated with the following equation:

$$20.13(\Delta A \; 646 - 710) - 5.03(\Delta A \; 663 - 710)$$

Chlorophyll A+B was calculated from the extract, and then normalised to the weight of the sample in mg to determine the concentration of chlorophyll with the following calculation, where V is the volume of acetone extract in the cuvette:

$$\frac{(27.8(\Delta A \; 710 - 652)) \; x \; V(ml)}{FW(mg)}$$

Gene expression analysis

For qRT-PCR experiments, seedlings harvested in liquid nitrogen were ground into fine powder. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion. cDNA synthesis was performed using the qScript cDNA SuperMix (Quanta Biosciences) as described by the manufacturer. The qRT-PCR was set up as a 10μL reaction using SYBR Green (Roche) in a 384-well plate, performed with a Lightcycler 480 system (Roche). Results were analysed using the Light Cycler 480 software. Unless otherwise stated, all experiments were performed in triplicate with 50 seedlings per replicate. All primers used in this study are as follows:

Primer name	**Sequence (5'-3')**
AG_2236_BCAT2_F	GGGATAATCTCGGGTTTGGT
AG_2237_BCAT2_R	CTTCATCCGGATAGCGTTGT
AG_2232_THDP_F	GACGAAGACGGACGAATCAT
AG_2233_THDP_R	TGCTGAAGCGATGTTAATGG
AG_2234_DIN2_F	CGGTCGTCGGAGAGAGTAAC
AG_2235_DIN2_R	GCCTTGCAAAACACCAAAAT
AG_2238_MCCA_F	CCCGTCTACAGGTCGAACAT
AG_2239_MCCA_R	ACCCGAACTGATGGTGAGAC
AG_2240_IVD_F_	ACTCTGTTGCGAGGGACTGT
AG_2241_IVD_R	CTTAGAAGGCGTCCTGTTGC
AG_2242_MCCB_F	CTTTGCCTTCAGGTGGGATA
AG_2243_MCCB_R	ACCGAGCAGCAATCTCTTGT
DY_1166_HXK1_F	GGTTTCACTTTCTCGTTTCCTG
DY_1167_HXK1_R	CTTGTCCAACTGCTTCTTCG
AR027_PP2A_F	TAACGTGGCCAAAATGATGC
AR028_PP2A_R	GTTCTCCACAACCGCTTGGT

JJF-1519-PIF7-F	CTATGGGTAACAGAGACTGCAC
JJF-1520-PIF7-R	TCCATATTCATTGTCTGTGCGA
AR077_HFR1_F	TGGCCATTACCACCGTTTAC
AR078_HFR1_R	AAACCGTGAAGAGACTGAGGAG
AR073_PIF5_F	CAAAGCCAAGCTCGTAGAGAAC
AR074_PIF5_R	GAGGTGATCGATCGAAGAGAAG
BBX15 FP qPCR	CTTGAGGAGTTTGCTGCTGACG
BBX15 RP qPCR	CCCTAGCTCCTCCATGGCATAC
EEL FP qPCR	GCTTGTTCGTCAGGGAAGCTTG
EEL RP qPCR	ATTTCACCGAGTGTAGGCTGCT
BBX23 FP qPCR	ACATCACCGAGTCGCCTTACaa
BBX23 RP qPCR	ACCCTTTTCTCTCCTGGCAGAT
OLEO2 FP qPCR	GGTCTAACCGGGCTTAGCTCAA
OLEO2 RP qPCR	CTGGCCCATTTCTTTGCCCTTT
PIL6 FP qPCR	GGGTAACAAATCGAGCCAACGG
PIL6 RP qPCR	ATCTGTTCTGCTGCAGTGAGGT
HKL1 qPCR F	CACTTGACCGCCGGTAAGAAAC
HKL1 qPCR F	CACTTGACCGCCGGTAAGAAAC
HKL1 qPCR R	CAGCTGTCACTGCTCTACCTGT
HXK2 qPCR F	GAAAGAGTTGGGCTGGACATGC
HXK2 qPCR R	GGTGCCCAAAATAACTGCGACA
HXK3 qPCR F	GCCATAAGCTTGCAAGTCACGT
HXK3 qPCR R	TAAATGGAGTTAGTGGCCGCCA
HXK4 qPCR F	TCGTTTGCGATGAAATGGAGCC
HXK4 qPCR R	AAGCAAACGCCTTTACGACGAG
QQS-F qPCR	ATGAAGACCAATAGAGAGCAGGAAA
QQS-R qPCR	AGTAGTTGTAGAACTGAAGCCCGAC
SYP111 FP qPCR	AGGGGCTGAGGCAAAAGATGAT
SYP111 RP qPCR	GCTCGCGTGAGAAACTCTTCAC
CDC20.1 FP qPCR	TCCAAAGAGGCCTACAGGAAGC
CDC20.1 RP qPCR	AGAGTGATTGCTGGGAAGCAGT
MAD2 FP qPCR	CGAATGGCTTGAAGCTGGGAAG

MAD2 RP qPCR	TCTCCCTCGACACACCTTTGTC
KINESIN-12B FP qPCR	TGCTCGGAGAAGAGGTTCCTTG
KINESIN-12B RP qPCR	CGTCCACTGACTCAGGAATGGT
CDT1B FP qPCR	ACAGTCATCACGAAGGAGGAGC
CDT1B RP qPCR	CAATCCGGGACCAATTGCAACA
CYCB1;1 FP qPCR	ATGGACCAGCACTCTCAAGCAT
CYCB1;1 RP qPCR	CTGATACGCCAACAGCTTTGCA
CYCA1;1 FP qPCR	TCGGCGATCTTCGTTTTCCTCT
CYCA1;1 RP qPCR	GGTGCTCGTTTCTTCGTCATCG
DEL1 FP qPCR	GCGATCTGAACCGAGCACAAAA
DEL1 RP qPCR	GCGATCTGAACCGAGCACAAAA
AUR1 FP qPCR	AACACCAGGAGAAGGAGGCTTC
AUR1 RP qPCR	ATTGCTCCGTTTTTCTCTGGCG
AUR2 FP qPCR	AGCAAGAGAGAAACGGAGCGAT
AUR2 RP qPCR	GAGCCGCAGAATATTGGGATGC
SIG5 FP qPCR	TGTGCTCGATTCATTGAAGCCG
SIG5 RP qPCR	TCTCCTAGAGTCCTGTCGCCTT
SIG4 FP qPCR	GTCTCAAAATGGCCGCATGACA
SIG4 RP qPCR	TTCACCATCTCCTCTGGCCTTG

Primers were tested for efficiency in a dilution series using multiple kinds of cDNA for accuracy, and plotted on a line to determine if the primer is viable for use in qPCR.

Promoter analysis

Promoter analysis was performed using the open source iTAK software using the *Arabidopsis* Gene Regulatory Information Server (AGRIS) to identify the predicted promoter region of HXK family genes. Binding sites were identified according to AGRIS references and colour coded.

Chapter 7. References

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Appendix A

The following text is a manuscript submitted for review.

HEXOKINASE 1 Promotes Post-Germinative Seedling Growth

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SUMMARY

Carbon availability and the light environment are strong determinants of seedling hypocotyl length. Under light limiting conditions, the glucose sensor HEXOKINASE1 (HXK1) and the PHYTOCHROME INTERACTING FACTOR (PIF) light signalling components are known to promote hypocotyl elongation. HXK1 is known to operate as a glucose-phosphorylating enzyme and as a glucose activated sensor-signalling molecule. Earlier work implicated HXK1 sensor-signalling in hypocotyl elongation, however, less is known of whether HXK1 enzymatic function, and/or HXK1-PIF pathway interaction are involved. We provide genetic evidence that HXK1-mediated glucose-phosphorylation is required for hypocotyl cell expansion in light limiting conditions. Application of glucose-6-phosphate, the HXK1 enzymatic product, restores short *gin2-1/hxk1-3* hypocotyls to wild-type length. Further, the HXK1 sensor-signalling complex components VHA-B1 and RPT5B do not contribute to this response. Unlike *gin2-1/hxk1-1*, the *vha-B1* and *rpt5b* alleles only disrupt hypocotyl growth following the application of exogenous glucose and not in more physiologically relevant conditions. mRNA-seq analysis illustrates that HXK1 and PIF signalling intersection at PIF5, HFR1, and other genes with known roles in light signalling. HXK1 imposes strong negative regulation on chloroplast and mitochondrial genomes, and also branched chain amino acid catabolism pathway genes which can provide a source of respiratory substrates in starvation conditions. Our study establishes the importance of HXK1 enzymatic function in supporting hypocotyl cell expansion, amino acid metabolism, and the transcriptional regulation of light signalling genes.

Key Words: Hexokinase, Phytochrome, Skotomorphogenesis, Hypocotyl, *Arabidopsis thaliana*

SIGNIFICANCE STATEMENT

This study highlights the critical role of the HXK1-G6P pathway in supporting post-germinative hypocotyl cell expansion. HXK1 moderates the availability of starvation-activated respiratory substrates, and the expression of the light signalling genes PIF5 and HFR1.

INTRODUCTION

The first days of life are critical for plant survival; successful seedling establishment relies on the mobilisation of seed reserves and the perception of light through wavelength-specific photoreceptors to support emergence before the switch to photo-autotrophic growth. HEXOKINASE 1 (HXK1), a metabolic enzyme with glucose-sensor signalling properties, and PHYTOCHROME-INTERACTING FACTORS (PIFs), play a pivotal role in coupling carbon availability to growth in the developing seedling (Rolland et al, 2002, Moore et al, 2003, Stewart et al, 2011, Lilley et al, 2012, Sairanen et al, 2013, de Wit et al, 2018, Krahmer et al, 2021).

In *Arabidopsis* and other oilseed plants, carbon resources are stored primarily in the form of triacylglycerols (TAGs) and seed storage proteins (SSP) which account for approximately 30-40% of the seed's dry weight each (Baud et al, 2008). After germination TAGs are degraded and converted to sucrose. TAG-derived fatty acids undergo B-oxidation, forming acetyl CoA, which fuels the production of sucrose via the glyoxylate cycle and gluconeogenesis. Sucrose is subsequently transported throughout the seedling to support development (Eastmond et al, 2000, Yang and Benning, 2018). In sink tissues, sucrose is cleaved by either SUCROSE SYNTHASE to produce UDP-glucose and fructose, or by INVERTASE to liberate glucose and fructose (Yoon et al, 2021). A vital step in the metabolism of these hexose sugars is their phosphorylation, which is mediated by HXK and FRUCTOKINASE (FRK). HXK can phosphorylate both glucose and fructose, while FRK primarily phosphorylates fructose. That said, FRK has a higher binding affinity for fructose than HXK, suggesting the principal role of HXK is the phosphorylation of glucose to glucose-6-phosphate (G6P) (Harrington and Bush, 2003, Claeyssen and Rivoal, 2007, Granot, 2007). In *Arabidopsis*, there are six different members of the HXK family: *HXK1-3*, and HEXOKINASE-LIKE (HKL) 1-3 (Karve et al, 2008). A key difference between the HXK and HKLs is the lack of glucokinase activity in the three HKL proteins, likely attributable to an indel at the adenosine binding site. Across the family, the most well studied member is HXK1.

In addition to its enzymatic role, HXK1 is known to operate as a glucose sensor-signalling molecule, directly coupling nuclear control of gene expression to glucose availability. Here, HXK1 forms a complex with partner proteins VACUOLAR H^+ ATPASE SUBUNIT B1 (VHA-B1) and REGULATORY PARTICLE 5B (RPT5B), one of two 19S Proteasome AAA-ATPases. Chromatin immunoprecipitation (ChIP) assays indicate VHA-B1- and RPT5B- dependent HXK1, enrichment at the 5'-272 BP region of the *CHLOROPHYLL A/B BINDING 2 (CAB2)* promoter (which contains DtRE, CUF-1, CCA1, and CGF-1 binding motifs). As none of the complex proteins possess DNA-binding domains, VHA-B1 and RPT5B are proposed to interact with transcription factors to regulate gene expression (Cho et al, 2006). Genetic analysis has shown that HXK1, VHA-B1 or RPT5B mediate glucose-induced repression of the photosynthetic genes, *CAB2* and *CARBONIC*

ANHYDRASE 1 (CAA) expression, and glucose-induced seedling developmental arrest (Cho et al, 2006). The retention of these responses in the HXK1 catalytic mutants G104D (Gly^{104} to Asp^{104}) and S177A (Ser^{177} to Ala^{177}), which retain the glucose-binding site, suggested in these instances, HXK1 operated through glucose-activated signalling, rather than through its enzymatic mode (Moore et al, 2003, Feng et al, 2015).

Interestingly, HXK1 glucose-signalling is not just implicated in *CAB2/CAA* expression and developmental arrest, which are elicited by high glucose levels, but also in the regulation of hypocotyl elongation in low light and low nutrient levels (Moore et al, 2003, Cho et al, 2006). The loss of function HXK1 mutants, glucose-insensitive 2 (*gin2-1)* (Landsberg erecta) and *hxk1-3* (Columbia-0) exhibit significantly reduced hypocotyl sizes, particularly in limited light conditions (Jang and Sheen, 1997, Jang et al, 1997, Kelly et al, 2021). Additionally, the *vha-B1* and *rpt5b* mutants phenocopy the *gin2-1* short hypocotyl phenotype, while the catalytically inactive alleles $hxk1^{G104D}$ and $hxk1^{S177A}$ restore the Wt hypocotyl response (Moore et al, 2003; Cho et al, 2006). It would be interesting to establish whether HXK1 catalytic action assists with the metabolism of seed reserves that fuel seedling growth prior to the establishment of photosynthetic competence.

The regulation of hypocotyl elongation in darkness and low light conditions is known to be regulated by PHYTOCHROME INTERACTION FACTORS (PIFs), a family of basic helix-loop-helix (bHLH) transcription factors. PIFs (PIF1, 2-8) are regulated by the phytochrome (phy) family of photoreceptors, of which phyB has been seen to be critically important in regulating seedling de-etiolation (Franklin and Quail, 2010). Excitation of the phyB chromophore with red (600-700 nm) light, induces a conformational change from the inactive (Pr) to the active (Pfr) form, which preferentially binds to PIF. This physical interaction results in rapid phosphorylation and subsequent degradation of PIFs 1, 3, 4, and 5, as well as sequestering them from their target promoters (Leivar et al, 2008, Park et al, 2012, Park et al, 2018). As a result, these PIFs are most active in darkness and low light, evident in the short hypocotyl phenotype of the quadruple *pifQ* mutant, which comprises *pif1-1, pif3-1, pif4-2,* and *pif5-3* alleles (Leivar et al, 2008). Thus, light-activated phyB repression of PIFs is critical for the switch from skotomorphogenic to photomorphogenic growth.

Both phy and PIFs have been implicated in carbon resource partitioning, where they have an important role in adjusting growth allocations to sink tissues in darkness and shaded conditions (Yang et al, 2016, de Wit et al, 2018, Krahmer et al, 2021). PIFs are involved sucrose regulation of hypocotyl growth; the *pifQ* mutant is insensitive to sucrose supplementation, and PIF5 protein stability is moderated by sucrose. (Liu et al, 2011). PIF4 has an important role in coupling carbon status to temperature-dependent hypocotyl growth (Hwang et al, 2019, Bian et al, 2022). Here, Trehalose-6-phosphate (Tre6P), a key indicator of sucrose status, stabilises PIF4 by inhibiting SNF1-RELATED PROTEIN KINASE1 (SnRK1)-mediated PIF4 phosphorylation and 26S proteasome

destruction. Further, phys, together with the nutrient-sensing pathways have been shown to have a critical role in stimulating seedling shoot apical meristem stem cell division, by activating TARGET OF RAPAMYCIN (TOR) (Pfeiffer et al, 2016). More recently, a link has been made between guard cell-located PIF4 and HXK1 and sucrose-mediated hypocotyl elongation in long days. This work demonstrated that HXK1 overexpression in guard cells enhances *PIF4* expression and hypocotyl elongation in response to blue light wavelengths (Kelly et al, 2021).

In this study, we assessed seedlings under darkness and low light regimes to determine HXK1 contribution to hypocotyl elongation fuelled by seed reserve utilisation. Genetic, pharmacological, and molecular analysis point to an important role for HXK1 enzymatic function in supporting hypocotyl growth in light-limiting conditions. Our mRNA-seq data reveals HXK1 exerts wide-ranging control of metabolic genes, including a branched chain amino-acid pathway, which provides an alternative source of carbon in starvation conditions. The mRNA-seq analysis also identifies HXK1 and PIFs signal convergence at key light signalling components. Finally, we demonstrate that several aspects of the HXK1 sensor-signalling pathway do not operate in light-limiting conditions.

RESULTS

HXK1 supports post-germinative hypocotyl growth in a G6P-dependent manner

The *gin2-1* mutant was previously reported to have impaired hypocotyl growth in low nutrient/low light conditions (Moore et al, 2003), which led us to examine the fluence rate in which HXK1 operates. We established that the *gin2-1* (Ler) and *hxk1-3* (Col-0) hypocotyls were shorter in darkness and very low irradiance light but were indistinguishable from the Wt at fluence rates greater than 10 $\mu mol\, m^{-2}\, s^{-1}$ (Fig. 1a, b). This altered fluence rate response is similar to the quadruple *pifQ* (*pif1-1, pif3-1, pif4-2, pif5-3)* mutant, though less severe. Interestingly, *gin2-1* and *hxk1-3* seedlings also have short hypocotyls in 8h light (L), 16h dark (D) short days and in longer photoperiods (16h L, 8h D), but only when the light fluence rate is low (Fig. S1). Thus, the *gin2-1* and *hxk1-3* long hypocotyl phenotypes are evident in darkness and conditions when light availability is restricted. We also observe similar reductions in the cotyledon area when light is limited (Fig. 1c).

To establish the cellular basis of the hypocotyl defect, we measured the epidermal cell length in the basal, middle, and upper regions of the hypocotyl, and recorded the total cell number in epidermal cell files (Fig. 1d, e). Our results show that *gin2-1* has a lower cell file count and significantly shorter epidermal cells through the hypocotyl. As hypocotyl cell number is largely determined during embryo development, this suggests a role for HXK1 at this early stage. As hypocotyl elongation is driven by hypocotyl cell expansion (Gendreau et al, 1997), our data indicates that HXK1 action may be required to promote epidermal cell expansion post-germination, particularly when light conditions are limiting.

Previously, HXK1-dependent hypocotyl growth was shown to result from glucose-induced nuclear signalling, rather than HXK1 enzymatic function (Moore et al, 2003, Cho et al 2006). To explore this further, we examined the hypocotyl response over a range of glucose concentrations in low-irradiance light and in darkness. As expected, in Wt the glucose response is biphasic, with lower concentrations promoting, and higher concentrations inhibiting hypocotyl growth (Singh et al, 2017) (Fig. 1f). In comparison, *gin2-1* and *hxk1-3* mutants exhibited reduced sensitivity to glucose, with a lagging dose-response curve (Fig. 1f, Fig. S2a, Fig. S2e, S2g). This was not observed in mannitol controls in both darkness and low light (Fig. 1f, Fig. S2c-d, i-j). The results indicate that HXK1 mutants have an altered hypocotyl dose response to exogenously applied glucose. Next, we applied a concentration range of glucose-6-phosphate (G6P) which is the product of HXK1 enzymatic activity. Contrasting with the glucose response, the Wt was largely insensitive to G6P. Further, we found that rising doses of G6P sequentially increased hypocotyl length in *gin2-1*, and *hxk1-3,* restoring to Wt length at 5mM (Fig. 1g, Fig. S1). Similar results were obtained for seedlings supplied with competitive HXK1 inhibitory molecule, D-mannoheptose, and 5mM G6P (Fig 1h). This suggests that in light-limiting conditions, and the absence of exogenous glucose, HXK1 enzymatic activity is required to fuel hypocotyl growth. In support of this proposition, we established that G6P application to *hxk1-3* successfully restores epidermal cell length in the middle and upper regions of the hypocotyl (Fig. 1i). As post-germinative hypocotyl cell elongation proceeds in a basal to distal wave, this suggests a potential window of sensitivity to G6P (Boron and Vissenberg, 2014). Thus, in light-restricted conditions, HXK1 enzymatic activity appears to play an important role in promoting hypocotyl elongation post-germination. In comparison, hypocotyl epidermal cells of *pifQ* mutants are much shorter than Wt and *hxk1-3* but are completely insensitive to G6P (Fig. 1i). This data indicates that PIF control of hypocotyl cell expansion is not due to deficiencies in G6P.

mRNA-seq reveals a role for HXK1 in translation and carbon stress metabolism

To gain a more in-depth understanding of the HXK1 function in seedling establishment, we performed an mRNA-seq on 4-day-old etiolated Ler and *gin2-1* plants. Briefly, total RNA was extracted, and 6 stranded libraries were prepared from polyA purified RNA. These were pooled and sequenced on an Illumina HiSeq 4000 platform. Gene counts were extracted using the ASpli R package (Mancini et al, 2021) and the AtRTDv2 annotation (Zhang et al, 2017). Raw counts were filtered to remove lowly expressed genes, normalized to library size and gene expression was quantified using EdgeR (Robinson et al, 2010). This resulted in 21,256 genes (62% of the 34,212 annotated AtRTD2 genes) being considered for downstream analysis. Using custom R scripts, we performed differential gene expression (DGE) analysis using EdgeR, followed by gene ontology (GO) enrichment analysis. We discovered 2,344 genes were significantly misregulated ($|logFC| > 0.58$, $p < 0.01$ and $FDR < 0.05$) in *gin2-1* compared to Ler, with a similar proportion of genes down (1,156) and upregulated (1,188) (Fig. 2a, Fig. S3). When we evaluated these genes, we found that the downregulated category of genes included lipid storage, and energy-consuming processes, such as

ribosome biogenesis and cell proliferation (Fig. 2b). In this figure, the bubbles represent each GO term, the size of the bubble indicating the representation factor (RF) and the colour indicating the *P*-value score. In contrast, the upregulated category had strong enrichment for genes involved in the cellular response to starvation, cellular respiration, ATP biosynthesis, amino acid catabolism and photosynthesis. These patterns are consistent with a starvation-type response and a reduced capacity to catabolise sugars.

We noted that genes that were strongly upregulated in *gin2-1* included several enzymes involved in the catabolism of the branched-chain amino acid (*BCAA*) genes: *BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2* (*BCAT2), DARK INDUCIBLE 2 (DIN2),* AT1G21400 *(E1A1), ISOVALERYL-COA-DEHYDROGENASE (IVD),* AT1G03090 *(MCCA),* and *3-METHYLCROTONYL-COA CARBOXYLASE* (*MCCB)* (Fig. 2c). *BCAA* genes are often suppressed in carbon-rich conditions, and induced by stress or starvation, as they provide alternative substrates for respiration (Pires et al, 2016, Binder*,* 2010). Indeed, we also observe strong glucose-induced suppression of the majority of these genes in both the Wt and *gin2-1* (Fig. 2d). In most cases, the efficacy of glucose is greater in *gin2-1* than in Wt, indicating HXK1 has a role in antagonizing the glucose response (Fig. 2c). Our data also shows that, as for hypocotyl growth (Fig. 1g), G6P application to Wt has no significant impact on *BCAA* gene expression. However, G6P treatment in *gin2-1* is effective in restoring the BCAA expression to Wt levels. This indicates *BCAA* gene expression is regulated by the HXK1-G6P pathway.

Another feature of the transcriptomic data analysis is the extent to which HXK1 controls organellar gene expression, with 23.5% of mitochondrial and 82.8% of chloroplast genes upregulated in *gin2-1* (Fig 3a). In the mitochondria, we observe enrichment for genes regulating metabolic proton and electron transport; and in the chloroplast, we see enrichment in all genes except for 23 genes associated with vesicle formation and chromatin remodelling. Figure 3b shows qPCR verification for the chloroplast-encoded *RNA POLYMERASE SUBUNIT* genes *RPOA, RPOB, RPOC1* and *RPOC2*, required for chloroplast gene transcription. Given the dramatic effect on the chloroplast genome, we wanted to establish whether HXK1 affected seedling greening capacity. This we tested by growing seedlings in darkness for four days and then exposing them to 3 or 100 μmol m^{-2} s^{-1} white light for 1 hour to promote chlorophyll production. We observed *gin2-1* mutants have significantly higher chlorophyll (and protochlorophyllide) levels in darkness and following both light treatments, suggesting chlorophyll production is enhanced in HXK1-deficient seedlings where resources for growth may be more limited (Fig. 3c).

Transcriptomics data Indicate HXK1 and PIF operate through distinct pathways

As HXK1 and PIFs regulate hypocotyl elongation, albeit through different mechanisms, we wanted to establish if there was evidence for pathway crosstalk or convergence. By comparing transcriptomic data from our mRNA-seq with an etiolated, 4-day-old *pifQ* mRNA-seq dataset from Zhang et al (2013), we discovered that there are 527 genes commonly misregulated between the two mutants: 175 of which are misregulated in the same direction, and 352 which are misregulated in opposite ways (Fig 3d). Interestingly, for genes that are downregulated in *gin2-1 and pifQ (gin2-1/pifQ down/down)* we observe enrichment (42 genes, or 24% of genes with synchronous regulation) in lipid storage, while in the *gin2-1/pifQ* up/up category, there is significant enrichment (18 genes, or 10% of genes with synchronous regulation) in hypoxia-response genes (Fig. S3). Genes with opposing *gin2-1/pifQ* down/up regulation are enriched in GO-terms groups for translation, ribosome biogenesis and cell proliferation. In contrast, opposing *gin2-1/pifQ* up/down category genes include those involved in the cellular response to starvation and amino acid catabolism. Notably, this latter group includes the *BCAA* genes *BCAT2, DIN2, THDP, IVD, MCCA* and *MCCB* that we have identified as HXK1-G6P regulated (Fig. 2c), all of which show opposing regulation by HXK1 and PIFs (Fig. 3e). We also noted that the same up/down category included a number of transcription factors such as *PHYTOCHROME INTERACTING FACTOR 5 (PIF5)* and *LONG HYPOCOTYL IN FAR-RED (HFR1),* with pivotal roles in light signalling, *DIG-LIKE 4 (DIL4), ABA 5-BINDING PROTEIN 3 (AFP3),* and *G-BOX BINDING FACTOR 3 (GBF3*), involved in ABA signalling, *GRETCHEN HAGEN 3.12 (GH3.12),* the auxin conjugating enzyme, and the *SMALL AUXIN UPREGULATED RNA (SAUR)* genes *SAUR1 (AT4G34770), SAUR25 (AT4G13790)* and *SAUR42 (AT2G28085)* (Fig.3f). Thus, HXK1 has a role in repressing the expression of this group of transcription factors that are promoted by PIFs.

Our mRNA-seq data indicates that in light restricted conditions HXK1 has a role in controlling nutrient resource management, while our physiological data implicate HXK1-G6P function in stimulating hypocotyl growth. Previously mutant alleles for the *VHA-B1* and *RPT5B,* which form a nuclear complex with HXK1, were shown to restrict hypocotyl growth (Cho et al 2006). In agreement, we found *vha-B1* and *rpt5b* restricted hypocotyl elongation with glucose concentrations as low as 28mM. However, when grown on media without additional glucose supplement, *vha-B1* and *rpt5b* were indistinguishable from Wt, while the *hxk1-3* phenotype was retained (Fig. 4a). To ensure that this discrepancy was not due to experimental error, we were successfully able to reproduce the pattern of elongation found in Cho et al (2006) when grown in the same conditions as that study (Fig. S4). We also examined the *king1 (kinγ)* mutant allele for the SNF1-RELATED PROTEIN KINASE REGULATORY SUBUNIT GAMMA 1, which was recently identified as another interaction partner for HXK1 (van Dingenen et al, 2019), though neither treatment resulted in a hypocotyl growth defect. The data suggests HXK1 can regulate hypocotyl elongation through nuclear signalling, but in the absence of exogenous glucose HXK1 enzymatic function is required.

Earlier reports have implicated HXK1 (VHA-B1 and RPT5B) in glucose repression of *CAB2* and *CAA* expression, so we were interested to establish if this regulation was exclusively through the HXK1 glucose signalling route. In line with these studies, we observe HXK1-dependent repression of *CAB2/CAA* when seedlings are grown in the presence of 55mM (1% w/v), but not 28mM (0.5 % w/v) glucose (Fig. 4b). The 28mM and 55mM application doses lead to 6.5-, and 12.5-fold increases in internal when glucose levels, while a ~115mM (2% w/v) leads to a 28-fold increase compared to controls (Fig. 4c). Further, even the lowest concentration (28mM) greatly exceeds glucose levels in seedlings exposed to the higher light fluence rates of 300 or 600 μmol m^{-2} s^{-1}. We also show that *CAB2* and *CAA* expression is not substantially affected by *gin2-1* following G6P application. Our data, therefore, lends further support to the notion that *CAB2* and *CAA* are suppressed by HXK1-VHA-B1-RPT5B in response to exogenous glucose, but they are not targets of the HXK1-G6P pathway in a physiologically relevant glucose environment.

To further investigate the HXK1 enzymatic role in hypocotyl extension, we next examined the $hxk1^{S177A}$ substitution allele, which lacks catalytic activity (Moore et al, 2003). The expectation is that if hypocotyl length (without added glucose) is due to HXK1 enzymatic activity, then $hxk1^{S177A}$ would have a short hypocotyl phenotype. We found that on media without glucose $hxk1^{S177A}$ hypocotyls were indistinguishable from Wt, while $hxk1^{S177A}$ hypocotyls showed reduced growth compared to Wt plants when glucose is supplemented (Fig. 4d). As this finding is at odds with our other data, including the *vha-B1, rpt5B* mutant analysis (Fig 4a), we speculated that the effect of $hxk1^{S177A}$ may be masked by a HXK1 glucose-signalling brought about by elevated internal glucose levels. Previous studies have shown that loss of HXK1 catalytic (in *gin2-1*) activity leads to over-accumulation of glucose, which we have independently verified for *gin2-1* and *hxk1-3* under our conditions (Fig. 4e). Further, this is also the case for $hxk1^{S177A}$ that has 1.3-fold higher levels than Wt (Fig. 4f). Next, we reasoned that if the $hxk1^{S177A}$ hypocotyl elongation results from high internal glucose levels, then $hxk1^{S177A}$ would exhibit an altered glucose dose-response. In accordance with this hypothesis, our data shows $hxk1^{S177A}$ seedlings have an augmented dose-response that is consistent with elevated internal glucose levels (Fig. S5).

To consolidate our findings, we analysed the mutant for phosphofructokinase (*PFK1*), which controls a rate-limiting step in glycolysis (Mustroph et al, 2007). Our data shows that in low light the *pfk1* mutant phenocopies the *hxk1-3* short hypocotyl, and as expected *pfk1* is unresponsive to G6P but is rescued by the application of the glycolytic product, pyruvate (Fig. 4g). These results further reinforce the important role of the HXK1, and the glycolytic pathway in supporting hypocotyl growth in light limiting conditions.

DISCUSSION

HXK1-mediated hypocotyl elongation is facilitated through its enzymatic role

The seedling hypocotyl is an important model system in *Arabidopsis* for the study of signal convergence, and growth is an important readout of these signals (Oh et al, 2014, Singh et al, 2017; Ivakov et al, 2017; Chen et al, 2018). Post-germinative cell expansion requires strong coordination of these signals and is dependent upon the mobilisation of seed reserves coordinated with signalling responses (Penfield et al, 2004; Stewart et al, 2011). In this study, we have demonstrated that HXK1 plays an important role in this process via enzymatic activity; through seedling establishment, HXK1 operates as a master regulator of the plastome and controls multiple aspects of the metabolic starvation response. For the most-part HXK1 operates independently of PIF signalling, though a discrete gene set including HFR1 and PIF5 were identified as possible signal convergence points.

HXK1 has been recognised to have two distinct roles: as an enzyme which catalyses the conversion of glucose to G6P, and as a nuclear-located sugar sensor-signalling molecule (Moore et al, 2003; Cho et al, 2006). Its role in seedling hypocotyl development has previously been ascribed solely to its nuclear signalling capacity; however, our data shows that when glucose is not supplied exogenously, HXK1 enzymatic activity is necessary and sufficient to support hypocotyl growth. This becomes more apparent when light is limited, whether through a shorter light period in diurnal conditions or with reduced light fluences in both continuous light and diurnal cycles (Fig. 1a-b, Fig. S2). In contrast to glucose, where dose-response curves are qualitatively similar in Wt and *gin2-1*/*hxk1-3*, G6P selectively restores the *gin2-1*/*hxk1-3* short hypocotyl to wild-type length (Fig. 1f-g, Fig. S1). These findings emphasise the importance of HXK1 in the breakdown of seed-derived carbon reserves, which fuel hypocotyl expansion in light-limiting conditions prior to activation of the photosynthetic machinery. Sucrose production from triacylglycerols and storage proteins feeds into glycolysis, which in turn allows for the production of energy, carbon skeletons, and metabolites that are required for cell expansion and growth (Penfield et al, 2004, Graham, 2008). Supporting this notion, we observe HXK1-G6P mediated epidermal cell expansion in seedling hypocotyls (Fig. 1g, 1i, Fig. S1, Fig. 4g).

Earlier work has implicated PIFs in sucrose-dependent hypocotyl elongation and auxin gene expression, with PIF5 having a prominent role (Stewart et al, 2011; Lilley et al, 2012). We were therefore intrigued to establish whether PIFs had a broader role in the integration of light and HXK1 signals to control growth. Our analysis demonstrates that the short hypocotyl *pifQ* mutant is completely insensitive to G6P, suggesting that G6P is not a limiting factor for growth in *pifQ*. However, as *pifQ* only represents 4 out of the 8 *PIF* proteins in *Arabidopsis*, there is potential for other PIF-mediated growth deficiencies operating in a HXK1-G6P responsive way.

HXK1 regulates the carbon stress transcriptome.

When analysing mRNA-seq data we discovered that *gin2-1* has a transcriptome signature that is reminiscent of sucrose starvation. Earlier studies indicate sucrose starvation leads to strong upregulation of genes involved in sugar metabolism, photosynthesis, lipid metabolism, cellular respiration, and downregulation of genes involved lipid storage, cell proliferation and ribosomal processes (Contento et al, 2004, Nicolaï et al, 2006). Our study reports a qualitatively similar pattern in the transcriptome for *gin2-1* (Fig. 2b). Amongst the *gin2-1* upregulated genes are those implicated in branched-chain amino acid (BCAA) catabolism (Fig. 2b-d), an evolutionary conserved mitochondrial pathway which can be induced by intense carbon starvation (Binder 2010, Hildebrandt et al 2015; Heinemann and Hildebrandt, 2021). Activation of the BCAA pathway provides an

alternative source of respiratory substrates that can be utilised during carbohydrate starvation. As G6P supplementation specifically rescues the expression of these genes, our data indicates *BCAA* genes are regulated through HXK1-G6P induced signalling. Interestingly, the expression of *BCAA* genes is known to be directly regulated by SnRK1 kinase-bZIP complexes (Pedrotti et al, 2018). This evolutionarily conserved protein kinase complex acts to maintain homeostasis under nutrient-stress conditions. It will therefore be interesting to establish if SnRK1 kinase-bZIP complexes can be regulated by HXK1-G6P.

In light limiting conditions HXK1 exerts strong control over the plastome.

Our mRNA-seq data reveals a significant effect of HXK1 on the expression of non-nuclear genes. In dark grown seedlings 82.8% of the chloroplast and 23.5% of the mitochondrial genes were elevated in *gin2-1* (Fig. 3a-c, Table S2). While glucose treatment has been shown to attenuate chlorophyll degradation in darkness, our data shows HXK1 represses chloroplast gene expression in darkness, which, in turn, suppresses chlorophyll levels following exposure to light (Ueda et al, 2020). Indeed, the connection between chloroplast biogenesis and metabolism is well established; albino mutants show high misregulation in genes involved with starvation and mobilisation of storage energy, consistent with the notion of chloroplast genesis representing a switch of growth strategies to autotrophism (Grübler et al, 2017). With respect to mitochondria, sucrose starvation has been shown to cause structural changes, which result in impaired protein production despite unimpeded transcription activity (Giegé et al, 2005). Additionally, we have shown HXK1 represses the expression of mitochondrial genes involved in processes including charged particle transport, ATP synthesis, and oxidative responses. Oxidation in the mitochondria has been linked to metabolism, feeding back into metabolic processes under both abiotic and biotic stress responses to maintain signalling capacity (Vanlerberghe, 2013). The impact of *gin2-1* on these organellar transcriptomes most likely reflects the reality of carbon-resource deprivation, and the need to prime the cellular machinery to switch to photosynthesis rather than reliance on limited seed reserves to fuel growth.

HXK1 and PIF signalling converges at a small gene subset

Our mRNA-seq analysis identified small group of genes (22% of total *gin2-1* misregulation) that exhibit similar or opposing regulation by PIF and HXK1 (Fig. 3d-f, Fig. S3). We were interested to establish that *BCAA* genes, identified as HXK1-repressed genes (Fig 2a-d), were promoted by PIFs. Likewise, a number of genes implicated in light signalling including *HFR1, GH3.12* and *SAUR* genes, were antagonistically regulated by HXK1 and PIFs. This suggests that while HXK1 and PIF-mediated signalling is largely distinct, co-regulation does occur at a few key components and the mechanisms in promoting elongation through the regulation of these observed genes for HXK1 and PIFs are distinct from one another. Indeed, a recent study demonstrated such a connection between HXK1 and

PIF4 (Kelly et al 2021). The study showed HXK1 located in guard cells controls hypocotyl elongation through PIF4, in white and blue light. The authors suggest that in contrast to red light, blue light is not effective in driving photosynthesis, and therefore in blue light conditions sucrose derived from seed storage is a major carbon source. The study has strong parallels to our low light study where hypocotyl growth is largely fuelled by seed reserves. In both instances, HXK1 has an important metabolic role in phosphorylating the sucrose cleavage products, glucose and fructose. Like HXK1, PIF transcription factors are primed to operate under low light conditions, and thus offer a route to couple carbon resource management to growth prior to the establishment of photoautotrophic growth.

HXK1-dependent glucose signalling requires high amounts of glucose to operate.

Previous work has shown *VHA-B1* and *RPT5B* form a nuclear complex with HXK1, which directly couples glucose sensing to nuclear signalling and hypocotyl growth (Cho et al, 2006). In agreement with this notion, we observe a hypocotyl defect in *vha-B1, rpt5b* mutants, but only when exogenous glucose is supplied. In contrast the *gin2-1*/*hxk1-3* short hypocotyl phenotype is observed both with and without glucose (Fig. 4a). Collectively the data implies hypocotyl elongation can be controlled through HXK1 glucose phosphorylation and sensor-signalling pathways, however, prior to the establishment of photoautotrophic growth the enzymatic pathway necessarily dominates. These observations are backed by qPCR analysis of *CAB2* and *CAA1*, both HXK1/VHA-B1- /RPT5B-dependent glucose repressed genes. Interestingly, we only observed HXK1-mediated repression at 55mM glucose (which elevates internal glucose levels by 12.5-fold) and not the lower concentration of 28mM glucose (6.5-fold increase) (Fig. 4b). This suggests HXK1-dependency is only observed when glucose levels exceed normal physiological levels, which may be more indicative of a stress response. The ABA signalling genes *ABI4* and *ABI5* have been shown to have key roles in abiotic stress and the glucose signalling response (Dijkwel et al, 1997, Arenas-Huertero et al, 2000). As ABI4 is known to directly regulate the expression of *CAB2* it will be interesting to establish whether the HXK1-VHA-BI-RPT5B complex operates with ABI4 or other ABA signalling components.

Our study provides strong evidence for HXK1 control of hypocotyl growth via phosphorylation of glucose, yet counterintuitively, the $hxk1^{S177A}$ catalytically inactive mutant does not have the expected short hypocotyl phenotype in low light (Moore et al, 2003; Fig. 4d, S4). We therefore speculated that in a similar manner to *gin2-1* and *hxk1-3*, loss of enzymatic activity could lead to enhanced internal glucose levels. This would effectively activate glucose signalling and hypocotyl elongation, which would effectively mask any growth impairment caused by loss of enzymatic function (Fig. 4e-f,). Our analysis, which revealed elevated glucose levels in $hxk1^{S177A}$ and a shifted glucose dose-response, provided support for this hypothesis, offering a rational explanation for the $hxk1^{S177A}$ phenotype. Evidence for the importance of glycolysis, comes from genetic analysis demonstrating the glycolytic

enzyme mutant *pfk1-1* phenocopies *hxk1-3*, while both mutants are rescued by exogenous pyruvate application (Fig. 4g).

In conclusion, this study has shown HXK1 performs an important role in post-germinative hypocotyl growth, by enabling the consumption of seed reserves, prior to establishment of photosynthetic competence. By controlling the expression of enzymes in the BCAA pathway, HXK1 provides a route to generate alternative respiratory substrates when carbon resources are limited. HXK1 control of the plastome, provides a means to link photosynthetic machinery establishment to carbon needs. While HXK1 control of light signalling components provides a potential route to couple resource availability to the regulation of growth.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions, and treatments.

The wild-type *Arabidopsis thaliana* accessions used in this study are Landsberg erecta (Ler) and Columbia-0 (Col). Seeds for mutants *gin2-1* (Ler), *hxk1-3* (Col), *pfk1-1* (Col), *vha-B1* (Col, SALK_028728), *rpt5b* (Col, SALK_069366), and *king1* (Col, At3g48530) were obtained from The Nottingham *Arabidopsis* Stock Centre (NASC), UK. The *hxk1*^{S177A} seeds were kindly shared by Professor Ruth Stadler of the University of Erlangen-Nuernberg. For all experiments, seeds were surface sterilized with bleach and Triton X-100 sown on 0.5X MS plates (0.8% agar, pH 5.7) and stratified in darkness for 2-3 days at 4°C. All plants were grown at 18°C for 4 days after stratification unless otherwise specified.

Seedling hypocotyl length and cotyledon area measurements.

Images of seedlings laid flat on growth media were used to quantify hypocotyl length and cotyledon area using ImageJ (NIH, Maryland, USA) and Adobe Photoshop CS6 (Adobe, California, USA), respectively. For glucose-6-phosphate (G6P) (Sigma G7879), sodium pyruvate (Sigma P2256), and D-mannoheptose (Sigma M6909-1G) treatments, the required concentrations from filter sterilized stocks were added to sterilized media and seeds directly sown. Unless otherwise stated, all experiments were performed in triplicate with 40 seedlings per replicate.

Seedling hypocotyl cell counts and length measurements.

For determination of etiolated hypocotyl epidermal cell lengths/numbers, seedlings were mounted on slides with water and visualized using an Eclipse E600 Nikon DIC microscope at 20x magnification. Individual cell lengths from each section of the hypocotyl (basal, middle, upper) were measured using ImageJ and cell number per file was obtained by manually counting cells from the root emergence point to the apical meristem. Unless otherwise stated, all experiments were performed in triplicate with at least 20 seedlings per replicate.

Glucose and chlorophyll quantification.

Seedlings were harvested in liquid nitrogen, finely ground into a powder, and ethanol extracted thrice. Glucose was then quantified from these ethanol extracts using enzymatic degradation at 340nm wavelength and normalized to material fresh weight. Chlorophyll and protochlorophyllide were quantified from these extracts by absorption of A and B type chlorophyll at wavelengths of A646, 652, 663, and 710nm. Unless otherwise stated, all experiments were performed in triplicate with 50 seedlings per replicate

Gene expression analysis.

For qRT-PCR experiments, seedlings harvested in liquid nitrogen were ground into fine powder. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion. cDNA synthesis was performed using the qScript cDNA SuperMix (Quanta Biosciences) as described by the manufacturer. The qRT-PCR was set up as a 10µL reaction using SYBR® Green 1 480 Lightcycler® Master (Roche) in a 384-well plate, performed with a Lightcycler 480 system (Roche). Results were analysed using the Light Cycler 480 software. The primers used in this study are listed in Supplementary Table S1. Unless otherwise stated, all experiments were performed in triplicate with 50 seedlings per replicate.

cDNA library preparation and high throughput sequencing.

Total RNA was extracted from 4-day-old etiolated Ler and *gin2-1* seedlings (biological duplicates of 50 seedlings per replicate) as described above. Samples were then sent to Edinburgh Genomics (University of Edinburgh, UK) for QC check and sequencing. Briefly, quality check of the samples was performed using Qubit with the broad range RNA kit (Thermo Fisher Scientific) and Tapestation 4200 with the RNA Screentape for eukaryotic RNA analysis (Agilent). Libraries were prepared using the TruSeq Stranded mRNA kit (Illumina) and then validated. Samples were pooled to create 4 multiplexed DNA libraries, which were paired-end sequenced on an Illumina HiSeq 4000 platform (Machine name K00166, Run number 346, flowcell AHT2HKBBXX, lanes 5 and 6). On average 26.6 million 150nt PE reads were obtained for each sample.

Processing of mRNA sequencing reads.

Raw sequence reads were trimmed with cutadapt 2.8 (Martin, 2011) with default parameters and --a set to 'AGATCGGAAGAGC', to eliminate adapter contamination from the PE reads. Trimmed reads were aligned against the *Arabidopsis thaliana* genome (TAIR10) with HiSat2 v2.2.1 (Kim et al, 2019) with default parameters, except in the case of the maximum intron length parameter, which was set at 5000. Count tables for the different feature levels were obtained from bam files using the 'ASpli::readCounts()' function of ASpli package version 2.4.0 (Mancini et al, 2021) with custom R scripts and considering the AtRTD2 transcriptome (Zhang et al, 2017). Count tables at the gene level

presented a good correlation overall between replicates and samples (Table S2). Raw sequences (fastq files) used in this paper have been deposited in the ArrayExpress (Kolesnikov et al, 2015) database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7654.

Differential gene expression (DGE) analysis.

DGE analysis was conducted using custom R scripts for 21,256 genes whose expression was above a minimum threshold level (10 counts in 0.7 of samples in the smallest group that express the gene). DGE was estimated using the edgeR package version 3.36.0 (Robinson et al, 2010; Lun et al, 2016), and resulting P values were adjusted using a false discovery rate (FDR) criterion. Genes with $p < 0.05$, FDR < 0.05 and an absolute log2 fold change > 0.58 were considered differentially expressed. Volcano plots, calculation and plot of chromosomal distributions, and UpSet plots of differentially expressed genes (DEGs) were generated using R.

GO and KEGG metabolic pathway analysis.

Gene set enrichment and KEGG pathway enrichment analysis were performed using a combination of custom-written R scripts and the clusterProfiler package (Yu et al, 2012) version 4.2.2 of Bioconductor. The 21,256 expressed genes were used as the universe gene set. All KEGG pathways of *A. thaliana* were derived from the KEGG Pathway Database (http://www.kegg.jp; Kanehisa et al, 2012). Only the terms with $p < 0.05$ and FDR <0.1 were further considered. Bubble plots were generated using R.

Statistical analysis.

The statistical difference between two populations was tested by two-tailed, unpaired Student's t-test. To compare three or more populations, a one-way analysis of variance (ANOVA) followed by Dunnett's test (comparison against a control) was performed. All analyses were done using GraphPad Prism 7 (GraphPad Software) unless otherwise indicated.

Accession numbers and data availability.

Raw sequences (fastq files) used in this paper have been deposited in the ArrayExpress (Kolesnikov et al, 2015) database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7654. All custom R scripts are available at https://github.com/aromanowski/gin2_darkness. Alternatively, they are available upon request to the authors

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Author Contributions

M.J.L., A.G., A.R. and K.J.H. planned and designed the research. A.G designed and performed, and analysed the mRNA-seq experiment, A.R. conducted the bioinformatics analysis of the mRNAseq data. All other experiments were performed and analysed by M.J.L and A.G. All authors contributed to manuscript preparation.

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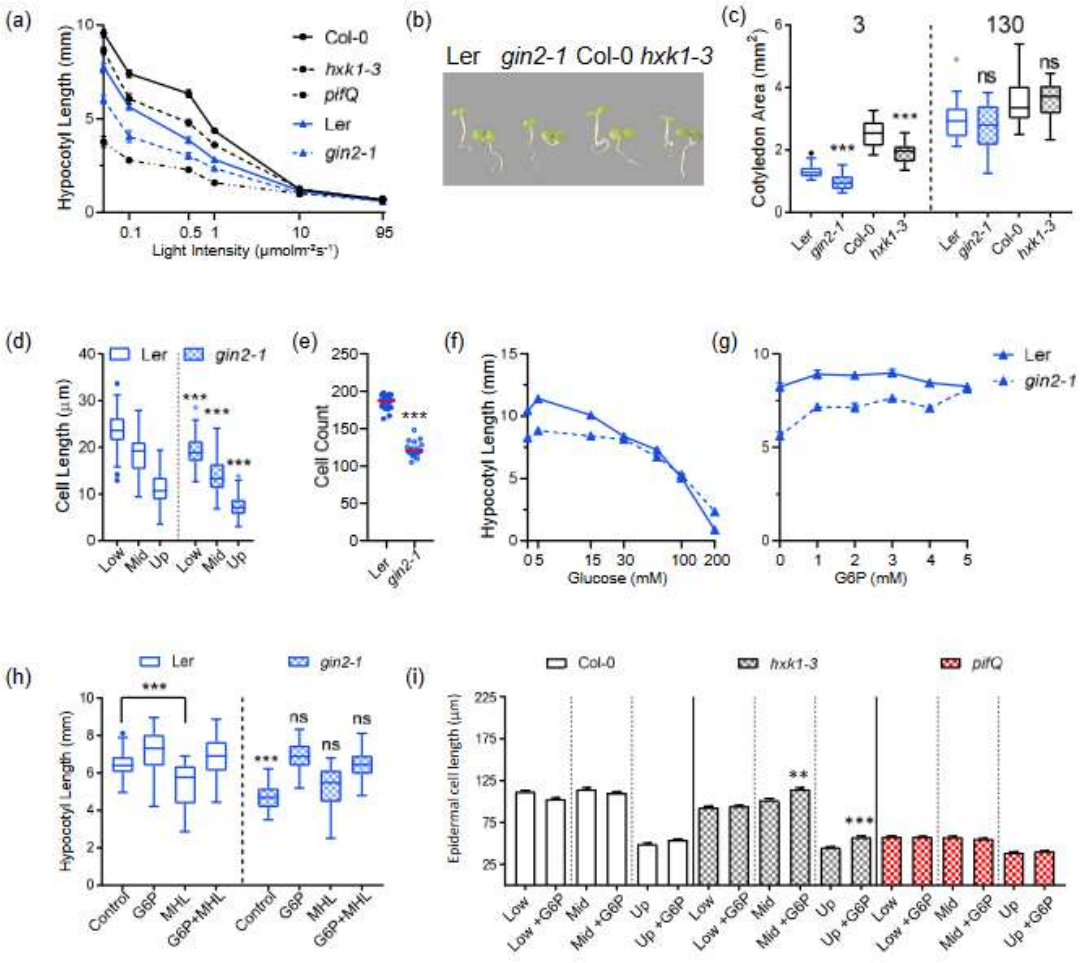

Fig. 1 Analysis of the HXK1-dependent hypocotyl growth phenotype and dependence on G6P production (a) White light fluence response. Seedlings under continuous white light in decreasing fluences. (b) Images of seedlings. Seedlings were grown in low light (left), or high light (right). (c) Cotyledon Area. Continuous light seedlings. Numbers above represent fluence rate in $m^{-2}s^{-1}$. (d) Cell size of *gin2-1* etiolated seedlings. Etiolated seedlings. (e) Cell file of *gin2-1* etiolated seedlings. Etiolated seedlings. (f) Glucose dose-response curve. Etiolated seedlings with increasing amounts of glucose. (g) G6P dose-response curve. Etiolated seedlings with increasing amounts of G6P. (h) Competitive treatment of HXK1 inhibitor molecule mannoheptulose and glucose-6-phosphate. Short day low light seedlings without or with G6P, mannoheptulose (MHL), and both. (i) Cell size and count of *pifQ* seedlings treated with G6P. Short day low light without (left) or with 5mM G6P (right).

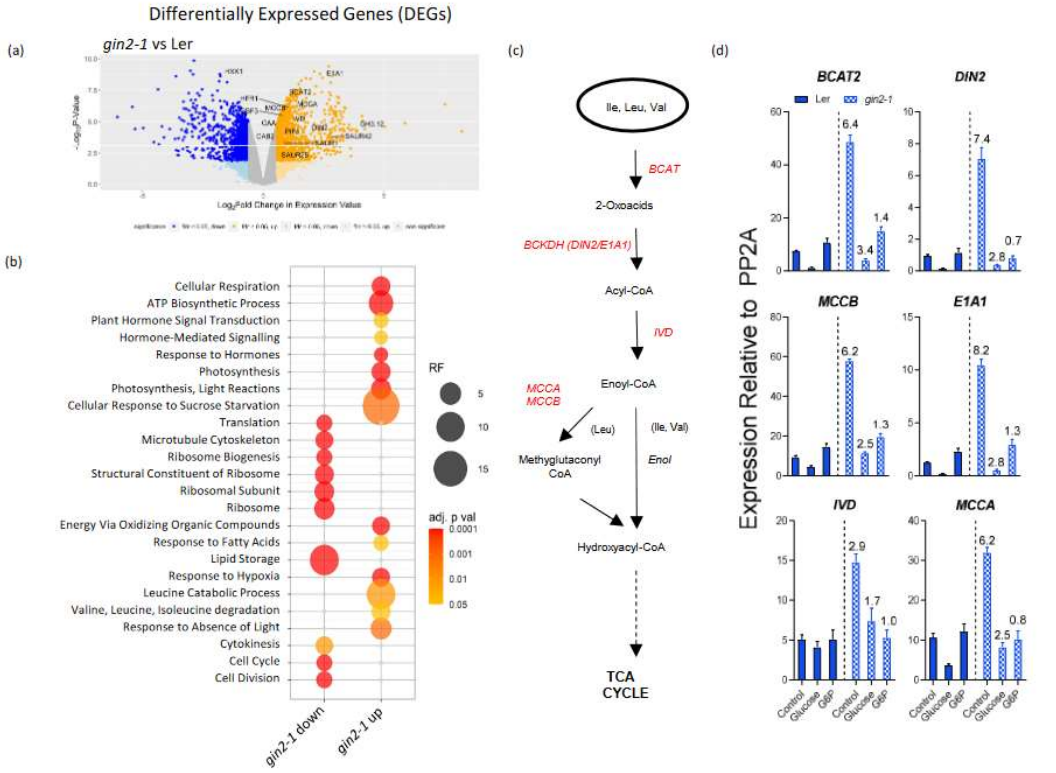

Fig. 2 *gin2-1* sequencing data reveals importance of HXK1 in translation and carbon stress metabolism (a) mRNAseq volcano plot. Yellow points represent logFC≥0.5 and blue points represent logFC≤-0.5, pval≤0.01. Red line marks pval≤0.01. (b) Bubble plot of selected GO terms in mRNAseq. Genes of interest were collected and displayed as described in the materials and methods. (c) Diagram of branched chain amino acid metabolism in Arabidopsis. Adapted from Neinast et al, 2018 with genes represented in (d) highlighted. (d) qPCR expression of core BCAA genes misregulated in *gin2-1*. Transcript abundance of BCAA pathway genes in etiolated WT and *gin2-1* seedlings grown with or without 0.5% w/v glucose or 0.125% w/v G6P. Numbers above represent fold-change between *gin2-1* and WT.

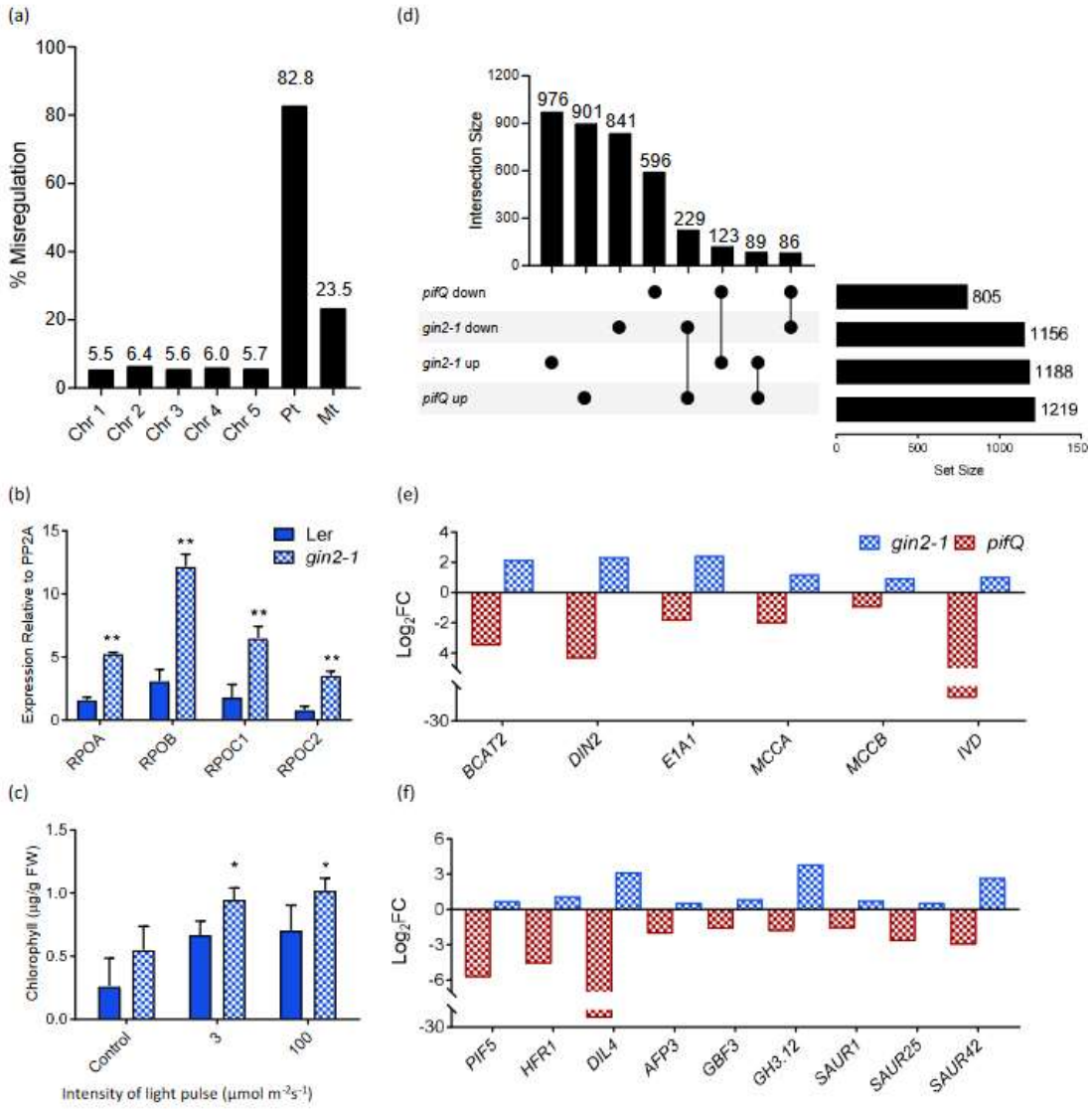

Fig. 3 mRNAseq of *gin2-1-1* implicate a significant role in regulating the plastid genome and demonstrate crosstalk with PIF-dependent signalling (a) Percentage of chromosome misregulation in *gin2-1*. Numbers above bar represent the percentage of genes significantly (P≤ 0.01) misregulated. (b) qPCR expression of RPO family genes, collected at ZT4. (c) Chlorophyll content of *gin2-1* and WT seedlings. Seedlings grown in darkness (left) or transferred to either 3 (middle) or 100 (right)100µmol $m^{-2}s^{-1}$ white light for 1 hour. (d) Transcriptome comparison between *gin2-1* and *pifQ*. Genes significantly misregulated in *gin2-1* were compared to genes significantly misregulated in Zhang et al (2013, were categorized as being either up or downregulated in either *pifQ* or *gin2-1* and sorted into overlap and exclusive subcategories. Numbers above the bars represent count. (e) BCAA genes representation in *gin2-1* and *pifQ*. (f) Genes of interest comparison between *gin2-1* and *pifQ* with published correlations with PIF. For all figures except (c), seedlings were grown in darkness.

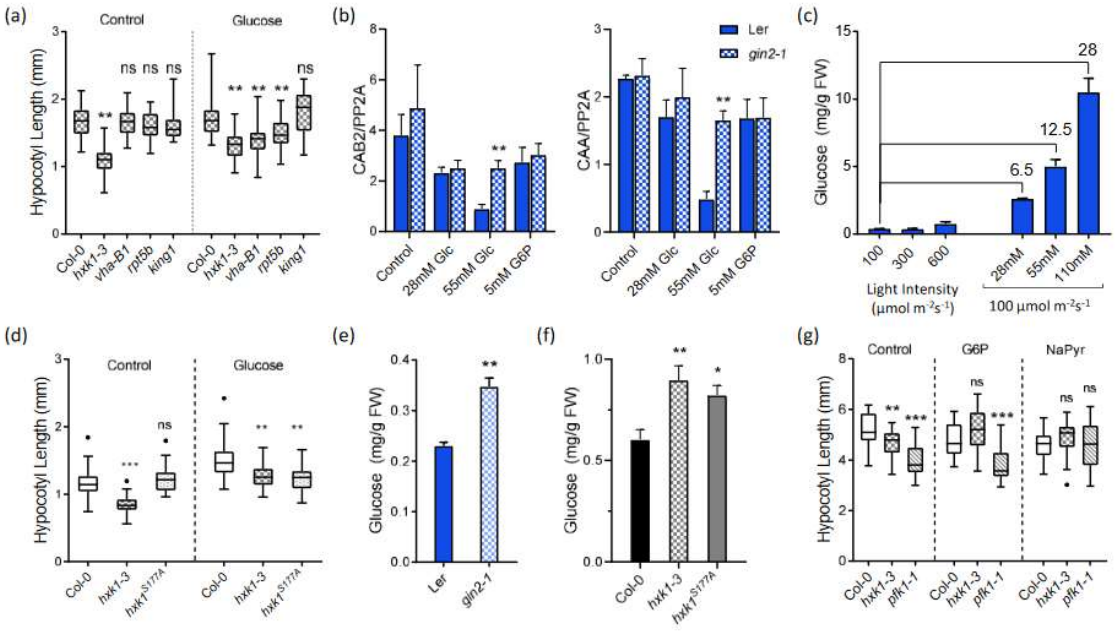

Fig. 4 HXK1-dependent glucose signaling requires high amounts of exogenous glucose to regulate expansion and genetic regulation (a) HXK1 signaling complex mutants grown without and with additional glucose. Short day seedlings in low light, without or with 28mM glucose. (b) Expression of CAB2 and CAA in *gin2-1* with added substrate. Transcript abundance of CAB2 (left) and CAA (right) relative to PP2A concentration in Short day seedlings in high light, without or with 28mM glucose, 55mM glucose, or 5mM G6P. Plants sampled at 4 hours after dawn (ZT4). (c) Internal glucose concentrations of Wt plants under variable light and nutrient conditions. Glucose measured at ZT 4 in Wt seedlings grown in 100, 300, and 600 µmol/m^{2}s, or at high light with increasing glucose. Numbers above horizontal bars represent the fold change. (d) *hxk1*^{S177A} seedlings grown with and without glucose. Etiolated seedlings without or with glucose. (e) Internal glucose concentration comparison between Wt and *gin2-1* seedlings. Glucose measured at ZT4 in etiolated seedlings. (f) Internal glucose concentration of *hxk1*^{S177A} seedlings. Glucose measured at ZT4 in etiolated seedlings. (g) Glycolysis component mutant *pfk1* grown without or with G6P or sodium pyruvate (NaPyr) in 3 µmol/m^{2}s WL.

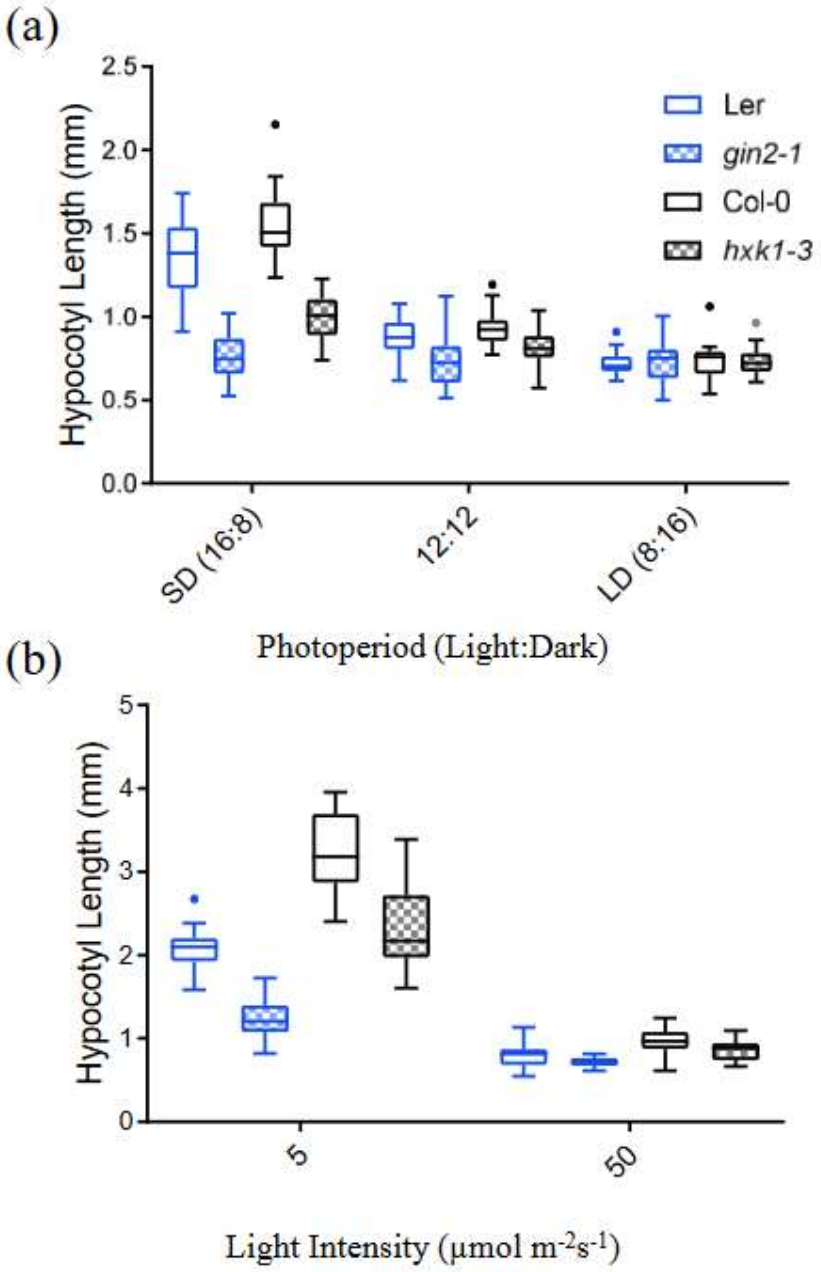

Fig S1. Diurnal growth of *gin2-1* and *hxk1-3*. (a) Effect of photoperiod on *hxk1* seedling growth. Diurnally grown seedlings grown in 100µmol $m^{-2}s^{-1}$ white light. (b) Effect of fluence rate on long day grown *hxk1* seedlings. Seedlings grown in long day.

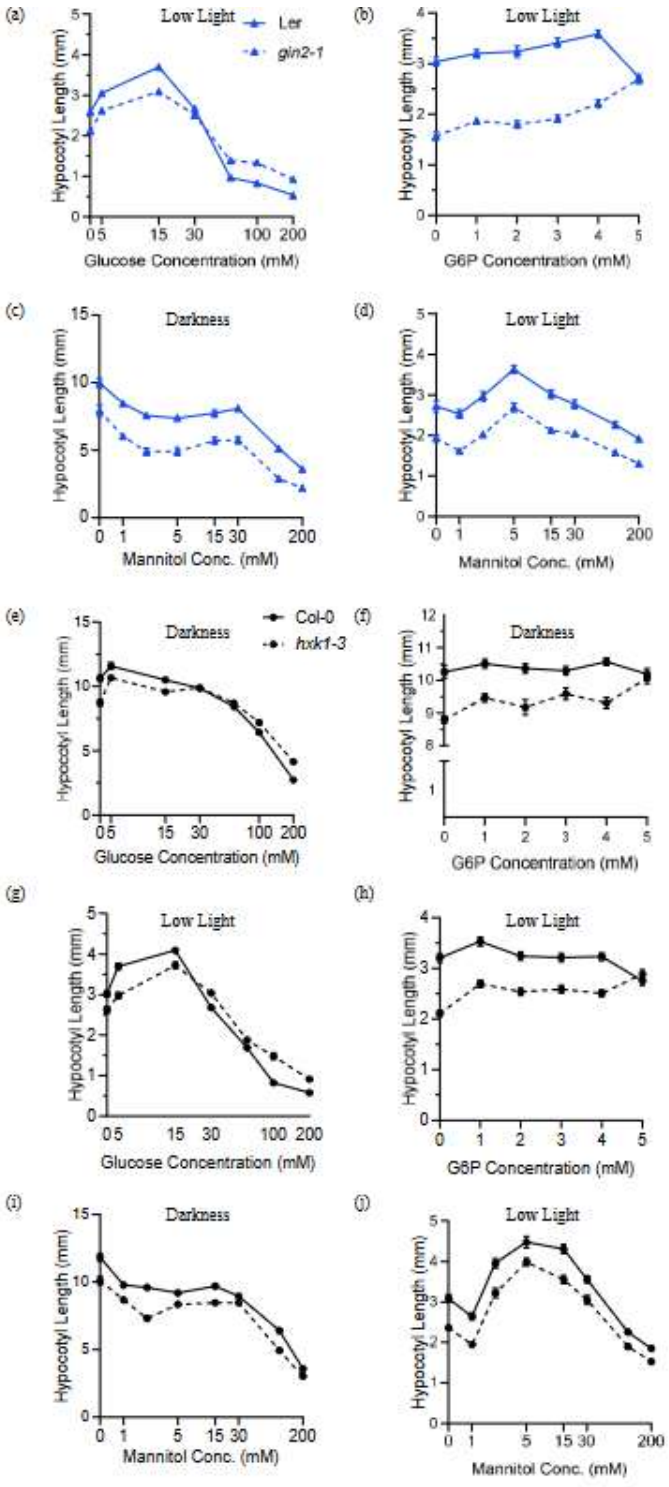

Fig. S2 Osmotic controls and low light treatment supports importance of G6P in HXK1-dependent hypocotyl elongation. (a) Low light glucose, Ler and *gin2-1*. (b) Low light G6P, Ler and *gin2-1*. (c) Darkness mannitol, Ler and *gin2-1*. (d) Low light mannitol, Ler and *gin2-1*. (e) Darkness glucose, Col-0 and *hxk1-3*. (f) Darkness G6P, Col-0 and *hxk1-3*. (g) Low light glucose, Col-0 and *hxk1-3*. (h) Low light G6P, Col-0 and *hxk1-3*. (i) Darkness mannitol, Col-0 and *hxk1-3*. (j) Low light mannitol, Col-0 and *hxk1-3*.

Category	Misregulated Genes	Total Expressed Genes	% Misregulation
gin2-1 up	1,188	21,256	5.59
gin2-1 down	1,156	21,256	5.44

Table S1. Proportions of genes represented in the mRNAseq in **Fig. 4a**.

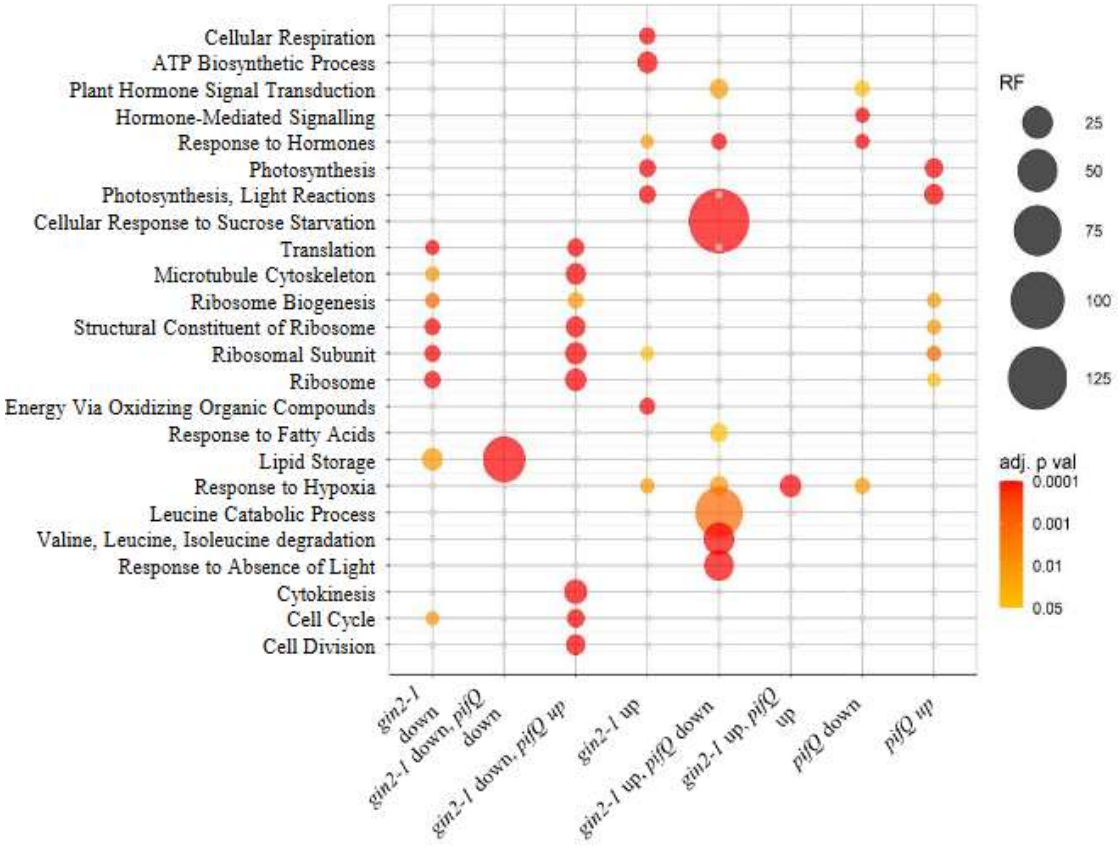

Fig. S3 Bubble plot of selected GO terms in *gin2-1* and *pifQ* comparative analysis. Genes of interest were collected and displayed as described in the materials and methods.

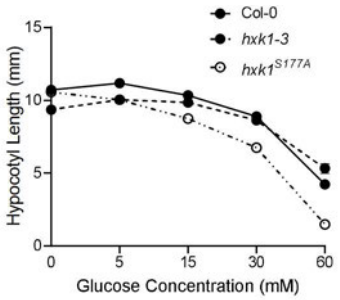

Fig. S4 Glucose dose-response curve of *hxk1*^{S177A} seedlings. Etiolated seedlings with increasing amounts of glucose.

Primer name	Sequence (5'-3')
AG_2250_RPOA_F	GCGATGCGAAGAGCTTTACT
AG_2251_RPOA_R	CCAGGACCTTGGACACAAA
AG_2252_RPOB_F	GATGTGAGGTGGGTTCAGAA
AG_2253_RPOB_R	GGTCTCCCGTCTTGCAAATA
AG_2254_RPOC1_F	TTCTTCCTCCCGAGTTGAGA
AG_2255_RPOC1_R	CCACGGCTTCTTGTACCAAT
AG_2256_RPOC2_F	CGCGTCGACTTGTTGAAGTA
AG_2257_RPOC2_R	CGTCTGCTAAGACACGACCA
AG_2236_BCAT2_F	GGGATAATCTCGGGTTTGGT
AG_2237_BCAT2_R	CTTCATCCGGATAGCGTTGT
AG_2232_THDP_F	GACGAAGACGGACGAATCAT
AG_2233_THDP_R	TGCTGAAGCGATGTTAATGG
AG_2234_DIN2_F	CGGTCGTCGGAGAGAGTAAC
AG_2235_DIN2_R	GCCTTGCAAAACACCAAAAT
AG_2238_MCCA_F	CCCGTCTACAGGTCGAACAT
AG_2239_MCCA_R	ACCCGAACTGATGGTGAGAC
AG_2240_IVD_F_	ACTCTGTTGCGAGGGACTGT
AG_2241_IVD_R	CTTAGAAGGCGTCCTGTTGC
AG_2242_MCCB_F	CTTTGCCTTCAGGTGGGATA
AG_2243_MCCB_R	ACCGAGCAGCAATCTCTTGT
AG_1267_CAB2_F	CCCTGGAGACTACGGATG
AG_1267_CAB2_R	TCCAAACTTGACTCCGTTCC
AG_1428_CAA_F	TGAATACGCTGTCTTGCACC
AG_1429_CAA_R	TGTGATGGTGGTGGTAGCGA
DY_1166_HXK1_F	GGTTTCACTTTCTCGTTTCCTG
DY_1167_HXK1_R	CTTGTCCAACTGCTTCTTCG
AR027_PP2A_F	TAACGTGGCCAAAATGATGC
AR028_PP2A_R	GTTCTCCACAACCGCTTGGT

Table S2. Primers used in the course of this study

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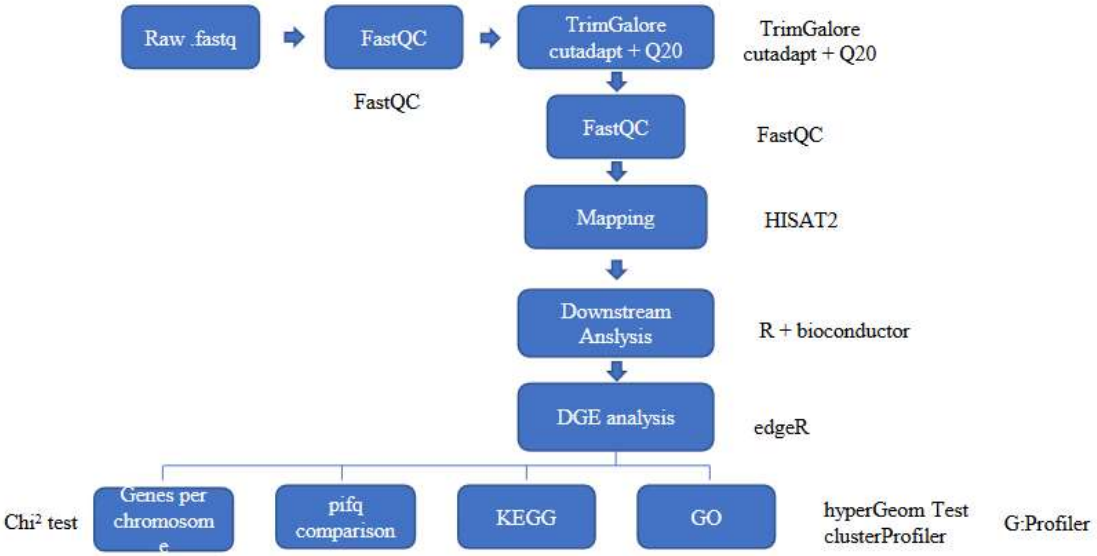

Fig. S6 Data analysis pipeline of mRNAseq

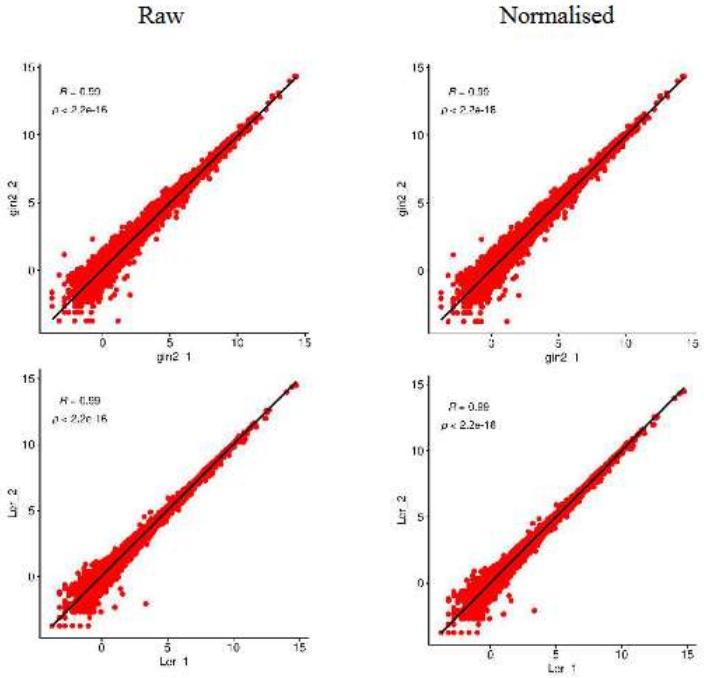

Fig. S7 Person sample-similarity correlation of *gin2-1* and Ler samples used in mRNAseq

Chromosome	Genes/ chromosome	Total Genes	Ratio	Total DEGs	Expected	DEGs/ chromosome	χ2 Values	Pval	$-Log_{10}P$
1	9701	3814	0.253993	2344	595.3591	530	7.18E+00	7.39E-03	2.13E+00
2	6312	3814	0.165262	2344	387.3731	407	9.94E-01	3.19E-01	4.97E-01
3	7624	3814	0.199613	2344	467.8917	427	3.57E+00	5.87E-02	1.23E+00
4	5842	3814	0.152956	2344	358.5288	353	8.53E-02	7.70E-01	1.13E-01
5	8419	3814	0.220427	2344	516.6816	478	2.90E+00	8.88E-02	1.05E+00
Pt	134	3814	0.003508	2344	8.2237	111	1.28E+03	2.70E-281	2.81E+02
Mt	162	3814	0.004242	2344	9.942085	38	7.92E+01	5.66E-19	1.82E+01

Table S3. Count tables at the gene level organised under chromosome and plastome. DEG=Differentially Expressed Genes.