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FUNCTIONAL CHARACTERIZATION OF HEXOKINASE-LIKE 1 (HKL1) FROM ARABIDOPSIS THALIANA

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FUNCTIONAL CHARACTERIZATION OF HEXOKINASE-LIKE 1 (HKL1) FROM ARABIDOPSIS THALIANA

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ABSTRACT

Arabidopsis hexokinase1 (AtHXK1) is a moonlighting protein with roles in both glucose signaling and catalysis. In this study, we first cloned and characterized the six HXK related genes from Arabidopsis. Three of the six encoded proteins were shown not to phosphorylate hexoses and thus, are designated as hexokinase-like (HKL) proteins. Though they are only 50% identical to HXK1, the amino acid sequences of HKL1 and HKL2 both have well conserved glucose binding domains and other recognized structural elements. The possible basis for their lack of catalytic activity was further probed by site-directed mutagenesis and ultimately was attributed to a suite of amino acid substitutions. Gene expression studies showed that transcripts of HKL1 and HKL2 occur in most plant tissues, thus supporting the hypothesis that they have regulatory functions.

The function of AtHKL1 was more closely examined using a reverse genetics approach. We identified a T-DNA knockout mutant for HKL1 and made HKL1 overexpression lines in different genetic backgrounds. Their phenotypes showed that HKL1 is a negative regulator of plant growth. Interestingly, many of the phenotypes required the presence of HXK1 protein. Both HKL1-GFP and HXK1-GFP are expressed at mitochondria and both were shown to interact with each other by coimmunoprecipitation assays. However, even though the HKL1 phenotypes included some dependence on glucose treatments, we conclude that HKL1 likely does not have a direct role in glucose signaling. Instead, we found from seedling signaling assays and a novel root hair phenotype that HKL1 mediates plant growth responses at least in part by

promoting ethylene biosynthesis and/or signaling. Overall, these studies have helped to identify, characterize, and define the function of non-catalytic HXKs from Arabidopsis.

DEDICATION

This work is dedicated to my wife, Rucha Karve whose constant inspiration lead me to pursue graduate studies. This work would not have been possible without her constant love and support.

Thanks for everything!

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Photosynthetic fixation of carbon and light energy in energy-rich sugar substrates by metabolic enzymes molecules is the most important process for sustaining the life on this planet. Sugars have been traditionally viewed as energy sources, as structural components and as food reserves. Sugars, in addition, have acquired important regulatory functions even early during evolution. Sugars are known to control metabolism, stress resistance, growth, and development in bacteria, yeast, plants, and animals.

In sessile organisms such as plants, the maintenance of energy homeostasis requires sophisticated and flexible regulatory mechanisms to account for the amazing physiological and developmental plasticity seen in plants. To regulate their growth and development, plants need to sense, transmit and respond to sugar signals together with internal hormonal signals and external cues such as light, temperature, biotic stress and abiotic stress. Several cross disciplinary approaches have been implemented to dissect the components of sugar signaling pathways in plants. Hexokinase (HXK), the gateway enzyme of the glycolytic pathway, has been shown to act as a glucose (glc) sensor in yeast, mammals and plants. Still, much needs to be learnt about the precise molecular mechanism of glc signaling in general and that of the role of HXK in particular. In this

review I will first discuss the process of glc signaling in yeast and mammals. I will then summarize our current understanding of plant glc signaling with an emphasis on HXK-dependent glc signaling. In the end I will elaborate on functional aspects of plant HXKs.

Glc sensing and signaling in yeast and mammals

Among all metabolizable sugars, glc has central metabolic importance in virtually all organisms, from microbes to humans. The initial step in glc metabolism is phosphorylation to form glucose-6-phosphate (glc-6P), the reaction catalyzed by HXK. Glc-6P can have multiple metabolic fates. Glycolytic metabolism of glc-6P is a major pathway for the generation of energy (ATP), and glycolytic intermediates also serve as precursors for the biosynthesis of other cellular constituents including amino acids and secondary metabolites. Metabolism of glc-6P through the pentose phosphate pathway generates NADPH and precursors required for a variety of anabolic pathways. Glc can itself be converted into polymeric forms used as support structures (cellulose) or carbohydrate reserves (starch and glycogen). Apart from these key metabolic functions, the pivotal role of glc as a signaling molecule is well illustrated in unicellular organisms like yeast and bacteria (Rolland et al. 2002).

Study of glc signaling in eukaryotes has been greatly aided by developments in the understanding of the glc signaling pathway in baker's yeast, *Saccharomyces cerevisiae*. The selective and rapid changes in gene expression enable yeast to survive adverse fluctuation of a key nutrient source. The process of glc sensing in yeast is

multifactorial, involving the role of hexose transporters (*HXTs*), HXK2 protein and signaling through cAMP (Rolland et al. 2002).

Among various glc-regulated genes, hexose transporter genes were found to be critical in glc sensing and signaling in yeast (Gancedo, 1998; Ozcan and Johnston, 1999). A number of yeast hexose transporters (HXTs) are transcriptionally regulated by multiple glc signaling pathways (Ozcan and Johnston, 1999). At least 16 of 48 carbohydrate transporter-like genes have been demonstrated to have transport functions while Snf3 and Rgt2, have been identified as sugar sensors that can bind to glc but are unable to transport glc. Upon binding to glc, the cytosolic, C terminus portions of Snf3 and Rgt2 interact with Yck Kinase and its substrates Mth1 and Std1, initiating a signaling cascade which ultimately activates transcription of target hexose transporters. This glc-mediated transcriptional regulation also controls sugar uptake in yeast. In the absence of glc, Rgt1 works as a transcriptional repressor and the association of Rgt1 with *HXT2* and *HXT4* promoter results in the repression of these HXT genes (Ozcan and Johnston, 1996). In contrast, in the presence of high glc, the association of Rgt1 with target HXT promoters is inhibited, thereby derepressing the transcription of target genes (Flick et al. 2003).

Glc phosphorylation in yeast is catalyzed by three enzymes, of which HXK2 is most active and is also required for glc repression of genes. The mechanism of glc signaling through HXK2 is yet unclear. There have been reports of a correlation between the catalytic activity and glc repression, but studies using site-directed mutations in HXK2 suggest that these two phenomena can be uncoupled (Hohmann et al. 1999). HXK2 was shown to interact with transcription factor Mig1 and thereby repress

transcription of target genes like invertase (Gancedo et al. 2008). HXK2 also is required for glc induction of certain glc transporter genes (Ozcan et al. 1996).

A third, glc signaling pathway in yeast is the cAMP pathway. In de-repressed yeast cells, glc triggers a rapid increase in cAMP, which initiates a protein kinase A (PKA) phosphorylation cascade. The downstream targets of PKA include enzymes involved in carbon metabolism such as neutral trehalase (Nth1) and 6-phosphofructo-2-kinase (Gancedo et al. 2008). In addition, glc induces activation of protein synthesis by PKA-dependent induction of ribosomal protein genes (Mager and Planta, 1991). This process stimulates cell growth and proliferation.

In mammals glc signaling is directly by hormones, unlike yeast where it is regulated by the metabolism. In humans, glc regulation requires adaptation to the external glc environment (nutrition) and the internal glc environment (blood glc). This is achieved largely by counterbalancing the secretion of glucagon and insulin by pancreatic α and β cells, respectively. Secretion of glucagon from α cells is increased when blood glc concentration is decreased, however the exact molecular mechanism underlying the glucagon release is not well understood (Cabrera et al. 2008). Pancreatic β cells respond to an increased rate of glc metabolism through glucokinase (GK) by increasing the secretion of insulin and by increasing insulin gene expression (Tarasov et al. 2004; Towle, 2005). Two transcription factors, PDX-1 and MafA, act synergistically and with the β-cell-specific factor BETA2 to promote insulin gene transcription (Zhao et al. 2005). When glc concentration is low PDX-1 is phosphorylated and moves to the nucleus (Macfarlane et al. 1999), similar to what occurs with the Mig1 repressor in yeast.

Understanding the link between changes in glc metabolism and activity of PDX-1 and MafA is essential for further understanding of this regulatory pathway.

The role of glc in altering gene transcription is understood best in the liver cells, where elevated glc concentrations increase the production of enzymes that are necessary for *de novo* lipogenesis (Towle, 2005). Promoters of genes encoding several lipogenic enzymes have been shown to contain two 6 bp motifs termed carbohydrate response elements (ChoRE). A 100 kDa bZIP/LZ protein was shown to bind to the promoters containing ChoRE (Yamashita et al. 2001). This ChoRE binding protein (ChoREBP) forms heterodimers with another bZIP/LZ protein Max like factorX (Mlx). MondoA, a paralog of ChoREBP also forms dimers with Mlx and activates transcription of a broad spectrum of metabolic genes, including those for the glycolytic enzymes (Sans et al. 2006). Interestingly, both ChoREBP and MondoA shuttle between the cytoplasm and nucleus in a glc dependent manner (Kawaguchi et al. 2001; Stoltzman et al. 2008). Nuclear translocation of MondoA depends on catalytic activity of human hexokinase (Stoltzman et al. 2008). Interestingly, the ability of human GK to complement glc-signaling defects of the yeast *hxk2* mutant indicates a potential signaling function of mammalian GKs (Mayordomo and Sanz, 2001).

Glc sensing and signaling in plants

Sugars affect plant growth and development directly by providing metabolic energy and indirectly by modulating other plant signal transduction pathways such as

those involving light, hormones, and nitrogen signaling (Smeekens, 2000; Coruzzi and Zhou, 2001; Leon and Sheen, 2003; Gibson, 2004). Some examples of sugar regulation of plant growth include the role of glc in cell cycle regulation (Riou-Khamlichi et al. 2000; Lorenz et al. 2003), seed germination (Pego et al. 1999; Price et al. 2003; Dekkers et al. 2004), cotyledon differentiation during embryogenesis (Borisjuk et al. 2004), flowering time (Ohto et al. 2001), senescence (Dai et al. 1999; Fujiki et al. 2001; Pourtau et al. 2006) and apoptosis (Kim et al. 2006). Before the initial observations of sugar regulation of gene expression in the late 1980's sugar effects on photosynthesis, growth and development were presumed to be the indirect result of the metabolic state. However, Sheen (1990) showed that glc, sucrose or acetate applied to maize mesophyll protoplasts led to repression of seven photosynthetic genes.

Sugar sensing and regulation in multicellular plants is undoubtedly a much more complex phenomenon than in the unicellular yeast for several reasons (Moore and Sheen, 1999). First, the plants use both long distance and intra-cellular signaling mechanisms to co-ordinate growth programs with environmental changes. As photosynthetic multicellular organisms, plants have distinct source (sugar exporting) and sink (sugar importing) organs. Photosynthesis and sink demand needs to be rigorously coordinated, and this coordination involves both metabolic (substrate and allosteric) regulation and specific sugar-signaling mechanisms (Rolland et al. 2006).

Although sucrose is the major photosynthetic and transport sugar in plants, various effects of sugars on growth and development can be attributed to glc or fructose, or their downstream metabolic intermediates (Koch 1996). A glc signal is generated by

different sources at different locations. In photosynthetic (source) cells, photosynthate generated in the Calvin cycle is exported during the day as triose-phosphates, from the chloroplast to the cytosol, for metabolism and/or converted to sucrose for export to sink tissue. In sink tissues, sucrose can be imported into cells through plasmodesmata (symplastic transport) or the cell wall/plasma membrane (apoplastic transport). Intracellular sucrose can be cleaved by cytoplasmic INV (C-INV), generating glc and fructose. The imported sucrose can also be stored in the vacuole, where it is hydrolyzed by vacuolar INV (V-INV). In the apoplast, extracellular sucrose is hydrolyzed by cell wall INV (CW-INV), generating extracellular glc and fructose, which are imported by hexose transporters (Rolland et al. 2006). In plants sucrose metabolism is characterized by a continuous process of degradation and biosynthesis of sucrose. This sucrose cycling has also been proposed to generate hexose signals (Moore et al. 1998). Excess photosynthate is transiently stored as starch in the chloroplast during the day and exported at night as maltose (Lu and Sharkey, 2004). This also could provide a major source for glc.

A number of genetic approaches have been used to define sugar-response pathways and to identify their components (Rook and Bevan, 2003; Leon and Sheen, 2003; Gibson, 2000). As a genetic model system, Arabidopsis has been used to isolate mutants from screens based on the observation that growth on media with high sugar levels can arrest seedling development. (Fig.1.1).

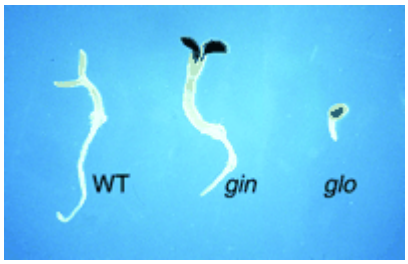

Figure 1.1 Arabidopsis seedling phenotypes on high glc medium. Wild-type (WT), glc-insensitive (*gin*), and glc-oversensitive (*glo*) plants were grown on 6% medium for 5 days under light (Figure was adapted from Rolland et al. 2002)

For example, Arabidopsis seeds can be exposed to high concentrations of glc that allow germination, but inhibit further seeding development. Mutants generated in this screen are *g*lc *in*sensitive (*gin*; Zhou et al. 1998). In contrast, *g*lc *s*uper *s*ensitive (*gss*; Pego et al. 1999) and *glc-o*versensitive (*glo*; Rolland et al. 2002) mutants also have been identified, but the affected genes in many of these cases have not been identified (Fig. 1.1). Similarly several mutants were identified by their phenotypic response to various other metabolizable sugars. Although the use of these genetic screens has led to identification of various components of sugar signaling, nonetheless many independently isolated sugar insensitive mutants are allelic to the mutants in various hormone signaling pathways.

One important issue within the study of signaling by any metabolite is that it is hard to distinguish an indirect metabolic effect from a direct signaling effect. As one approach, various sugar analogues have been used to overcome this problem. Among these, mannose is an epimer of glc known to be phosphorylated by HXK, but poorly metabolized. Mannose treatments have shown that glc metabolism beyond phosphorylation is not required to trigger sugar response genes (Graham et al. 1994; Jang

and Sheen 1994). Similar results were found using 2-deoxyglucose, a glc analogue phosphorylated by HXK, but also poorly metabolized. Other glc analogues, 6-deoxyglucose and 3-*O*- methylglucose, are transported into the cell but are not substrates of HXK, but did not trigger the repression these sugar response genes. However, expression of several plant genes, such as Arabidopsis patatin storage protein (Martin et al. 1997) is induced by sugar analogues that cannot phosphorylated. These results using glc analogues suggest that glc signaling in plants involves both HXK-dependent and HXK-independent pathways.

Based on the role of HXK1, three distinct glc signal transduction pathways are defined in plants (Xiao et al. 2000).

a. A HXK1- dependent pathway in which gene expression is correlated with the HXK mediated signaling function. A major effect of this pathway is regulation of photosynthetic genes.

b. A HXK1- independent signaling pathway. Glc induction of *CHS*, *PAL1* and genes encoding *AGPase* in addition to glc repression of *ASN1* are observed independent of sense and antisense overexpression of AtHXK1 or overexpression of yeast HXK2

c. A glycolysis dependent pathway in which gene expression is modulated by metabolites downstream of the sugar phosphates in glycolysis. This pathway has been suggested to regulate the pathogenesis related *(PR)* genes.

There are at least two HXK-independent signaling pathways in plants. Genetic and biochemical evidence supports the involvement of heterotrimeric G proteins in glc signaling. Arabidopsis genome encodes for one canonical Gα subunit (AtGPA1), one Gβ

subunit, and two Gγ subunits, but, as yet no GPCR. However, AtGPA1 is shown to interact with Regulator of G-protein signaling 1 (RGS1), an unusual hybrid seven-transmembrane domain protein with a C-terminal RGS-box (Chen et al. 2003). Based on the use of different glc analogs, it is suggested that AtRGS1 functions in an HXK independent glc signaling pathway (Chen and Jones, 2004). The *rgs1* mutant seedlings display insensitivity to the developmental arrest on 6% glc, whereas RGS1 overexpression lines are glc hypersensitive (Johnston et al. 2007).

Another potential extracellular glc sensing mechanism in plants may involve proteins analogous to the yeast glc transporter-like sensors, Snf3 and Rgt2 (Lalonde et al. 1999). Monosaccharide transporters with extended cytoplasmic loops similar to Snf3/Rgt2 are encoded in the Arabidopsis genome, and the monosaccharide sugar transporter STP13 acts as a heterologous multicopy suppressor of the yeast *snf4*Δ mutant growth phenotype (Kleinow et al. 2000).

A regulatory role of plant HXK in glc signaling was suggested initially by testing the effects of a variety of sugars, glc analogs and metabolic intermediates on photosynthesis and the glyoxylate cycle (Graham et al. 1994; Jang and Sheen 1994). Jang et al. (1997) used transgenic Arabidopsis plants with altered HXK1 expression to further examine sugar signaling. Plants with decreased HXK1 (35S::antisense-HXK1) expression were hyposensitive to glc induced seedling developmental arrest, whereas those over expressing HXK1 (35S::AtHXK1) were hypersensitive to glc induced seedling developmental arrest. Furthermore, yeast HXK2 could not compensate for the signaling

function of AtHXK. This indicated that the HXK catalytic and regulatory functions can be uncoupled (Jang et al. 1997).

Proof of HXK1 function as a direct glc signaling protein came from the work of Moore et al. (2003). Working with two independent null mutant alleles of HXK1 (*gin2-1* and *gin2-2*) the authors provided evidence of HXK1 as a sugar sensor in Arabidopsis. The *gin2* seedling could overcome glc induced seedling developmental arrest and the normally glc repressed photosynthetic genes *CAB, CAA* and *SBP* were derepressed in *gin2*. In addition to overcoming the seedling developmental arrest, different phenotypes of *gin2* also indicate that HXK1 promotes growth through interactions with hormone signaling pathways. These authors made transgenic Arabidopsis in *gin2-1* background with two catalytically inactive *AtHXK1* alleles, G104D and S177A, which are deficient in ATP binding and phosphoryl transfer, respectively. These transgenics could sustain WT growth but had GK activity equal to *gin2*. The G104D and S177A mutants could also restore photosynthetic gene repression to the levels detected in WT seedlings. This complementation of *gin2-1* with catalytically inactive HXK1 provided definitive proof of a distinct sensing function of HXK1 which is independent from its catalytic function.

Molecular mechanisms of glc regulation in plants

Available evidence indicates that glc regulation of gene expression involves different molecular mechanisms. Most studies on glc activation and repression of gene expression have emphasized the regulation of gene transcription. Short-term treatments

of Arabidopsis seedlings with glc or sucrose have been shown to affect the expression of about 1,000–1,700 transcripts, depending on experimental conditions (Price et al. 2004; Osuna et al. 2007). Studies with several maize photosynthetic gene promoters indicate that different regulatory elements are involved in sugar repression and negative control of positive *cis*-elements (Sheen 1990, 1999). Many sugar-regulated genes and promoters have been used to screen for Arabidopsis mutants with potential defects in transcription control. Baier et al. (2004) used the *APL3* (ADP glc phosphorylase large subunit-3) gene promoter fused with firefly luciferase gene to transform Arabidopsis. This screen based on the activity of a luciferase (LUC) reporter gene under the control of the *APL3* promoter yielded *high sugar-response* (*hsr*) mutants that exhibited elevated LUC activity and *APL3* expression in response to low sugar concentrations. Characterization of *hsr* mutants indicated that the regulation of sugar-induced and sugar-repressed processes controlling gene expression, growth, and development in Arabidopsis are affected in these mutants. A similar approach using β glucuronidase gene expression was used by Martin et al. (1997) for genes specific for sink tissue. The Arabidopsis β amylase gene is induced by sugars and mutants that display either increased or reduced sugar sensitivity have been isolated in amylase activity screens (Donggiun et al. 1998; Mita et al. 1997).

Different types of *cis* elements have been identified in sugar-regulated plant promoters: the SURE (sugar-responsive) (Grierson et al. 1994), SP8 (Ishiguro and Nakamura, 1994), TGGACGG (Maeo et al. 2001), G-box elements (Giuliano et al. 1988), B-box elements (Grierson et al. 1994; Zourelidou et al. 2002) and TELO motif (Li et al. 2006). TELO-motif was significantly enriched in the promoters of protein and nucleotide

synthesis genes. The combination of TELO and TEFF motif (Tremousaygue et al. 1999) in front of a minimal promoter driving GUS expression conferred glc-responsive expression in root meristems of transgenic Arabidopsis plants (Li et al. 2006).

Glc not only controls gene expression at the transcriptional level, but also at a post transcriptional level. In rice, glc affects *α-amylase* mRNA stability through a transcription-dependent mRNA destabilization process (Sheu et al. 1996). In Arabidopsis, short term treatment of seedings with glc results in disruption of F-actin fine filaments. Actin disruption also blocks the repression of glc regulated promoters in Arabidopsis (Balasubramanian et al. 2007). A targeted modulation of translational control has been proposed to play a role in this destabilization of the actin cytoskeleton by glc (Balasubramanian et al. 2007).

Protein stability and selective proteolysis have emerged as major regulatory mechanisms in plant signaling and development (Smalle et al. 2004). Glc signaling pathways also make use of these mechanisms to regulate downstream targets. Glc antagonizes ethylene signaling by enhancing proteasome-dependent degradation of the key downstream transcriptional regulator EIN3. Ethylene on the other hand, enhances EIN3 stability. Interestingly, this glc response is dependent on AtHXK1 (Yanagisawa et al. 2003).

Cellular context of HXK dependent glc signaling

Although it is widely accepted that HXK acts as a glc sensor in Arabidopsis, the cellular context of HXK dependent signaling is yet unclear (Balasubramanian et al. 2008). One suggestion is that HXK-dependent signaling in plants occur in an analogous fashion to yeast, in which nuclear localized HXK regulates transcription of target genes (Rolland et al. 2002). However, in plants there are conflicting reports on the sub cellular locations for this glc sensor. Heazlewood et al. (2004) reported from a proteomic analysis that AtHXK1 is one of 416 identified mitochondrial proteins from a dark-grown Arabidopsis cell culture. When expressed in tobacco (*Nicotiana tabacum*) protoplasts, AtHXK1-GFP expression was associated only with mitochondria (Damari-Weissler et al. 2007). On the other hand, Yanagisawa et al. (2003) reported that based on differential detergent extraction, AtHXK1 occurs at least in part in the nucleus of isolated protoplasts. Cho et al. (2006) using a similar biochemical fractionation presented evidence that a putative nuclear-localized AtHXK1 forms a glc signaling complex with two unconventional proteins, vacuolar H^+-ATPase B1 and a subunit of the 19S regulatory particle of the proteasome. The putative nuclear HXK complex was shown by using ChIP assays to bind to the promoter of *CAB2,* a known glc response gene. Even though the results of Cho et al. (2006) support a role of HXK1 as a regulator of promoter expression, there was no direct evidence presented to show that HXK1 does occur in the nucleus at anytime during plant growth.

Balasubramanian et al. (2007) on the other hand observed HXK1-GFP transiently expressed in Arabidopsis protoplasts as well as in stable transgenic lines. HXK1 was exclusively localized on mitochondria. Their results were further supported by the results

of organelle fractionation of Arabidopsis and pea leaf tissue, in which HXK1 was found only in the mitochondrial fraction but not in the nucleus. They also identified an interaction between HXK1 and porin, which indicates that porin is a mitochondrial docking protein for HXK1. AtHXK1 has an N-terminal 24-amino acid peptide that is predicted to function as a targeting sequence. The results of Balasubramanian et al. (2007) confirmed that this peptide is both necessary and sufficient for protein targeting to mitochondria in transfected leaf protoplasts. Further, unlike castor bean (*Ricinus communis*) endosperm in which mitochondrial HXK which is released by sugar phosphate treatment (Miernyk and Dennis, 1983), AtHXK1 did not move from mitochondria in response to ATP, ADP, G6P, F6P or light/dark treatments. These observations suggest alternative mechanisms that might account for different aspects of glc signaling such as translational and post translational regulatory processes.

Interaction of glucose signaling with other signaling pathways

An important factor that complicates the characterization of components of sugar signaling pathways is the fact that these pathways exhibit 'cross-talk' with many other plant response pathways. Both genetic screens based on seedling developmental arrest on high sugar media and those based on transgenic plants containing reporter gene constructs have yielded a large number of mutants allelic to known hormone signaling related mutants, predominantly in ABA and ethylene signaling. ABA was found to be of major importance for the ability of the germinating seeds to respond to sugar. Several

sugar signaling mutants contain lower levels of endogenous ABA than WT plants (Leon and Sheen, 2003) and exogenous glc has been shown to retard the rate at which endogenous ABA levels decline in germinating Arabidopsis seeds (Price et al. 2003).

Abscisic acid insensitive4 (*abi4*) mutant was originally isolated because of its ability to germinate on ABA containing media normally inhibitory for the wild type. The responsible gene was identified as an Apatela2 –transcription factor (Finkelstein et al. 1998). However, many of the sugar response mutants are allelic to *abi4* (*sun6,* Huijser et al. 2000; *sis5* Laby et al. 2000; *gin6,* Arenas-Huertero et al. 2000). Several mutants involved in ABA biosynthesis have also been isolated in sugar response screens, most notably different alleles of *aba2* (*gin1*, Zohu et al. 1998; *sis4* Laby et al. 2000). In addition, overexpression of Arabidopsis ABA-responsive element binding factors ABF3 and ABF4 confers both ABA and glc oversensitive phenotypes, supporting further interactions between glc and ABA signaling (Kang et al. 2002). Also, ABF2, an ABA response element (ABRE) binding basic leucine zipper (bZIP) TF, is an essential component of glc signaling (Kim et al. 2004). These studies show that germinating seedlings apparently require an intact ABA signaling pathway to carry out glc signaling (Smeekens 2000).

The loci shown to be associated with both ABA and sugar response have been limited so far to those encoding ABA biosynthetic enzymes (ABA1/ABA2/GIN1/SIS4) and ABI3/GIN5/LOS5), some ABI transcription factors (ABI4/GIN6/SIS5/SUN6) and related proteins (ABF3 and ABF4) (Leon and Sheen, 2003). Among these, the ABI transcription factors appear to function in the same signaling pathway mediating ABA

response (Finkelstein et al. 2002) and their expression is induced by glc in a HXK dependent manner (Leon and Sheen 2003) (Fig. 1.2).

Phenotypic and genetic analysis of the glc-insensitive, *gin1* mutant first revealed the interaction between sugar and ethylene-mediated signaling pathways (Zhou et al.1998). The observation that the glc insensitive phenotype can be mimicked by ACC treatment of WT seedlings prompted the investigation of possible interactions between glc and ethylene signaling (Zhou et al.1998). Similar to *gin*, the constitutive ethylene biosynthetic mutant *eto1* (ethylene overproducer) and constitutive ethylene signaling mutant *ctr1* (constitutive triple response) were found also to be insensitive to glc induced developmental arrest. In contrast, ethylene insensitive mutants, including *etr1, ein2, ein3,* and *ein6* also exhibit glc hypersensitivity (Zhou et al. 1998) (Fig. 1.2). Interestingly two other *Arabidopsis* mutants, *sis1* (Gibson et al. 2001) and *gin4* (Rolland et al. 2002) were originally identified in phenotypic screens for components of glc signaling, yet are allelic to *ctr1* (Kieber et al. 1993). However unlike *eto1* and *ctr1*, *gin1* does not display the ethylene triple response phenotype in the dark (Zhou et al. 1998). This phenotype indicated that the *gin1* phenotype and the triple response phenotype are uncoupled (Rolland et al. 2002). Double mutant analysis also suggest that GIN1 acts downstream of both ETR1 and HXK1 (Zohu et al. 1998). Further experiments have shown that *gin1* is allelic to *aba2,* a gene required for ABA biosynthesis (Cheng et al. 2002). This result suggests that glc can modulate ethylene signaling through the ABA pathway (Rolland et al. 2002). Further, EIN3 (ethylene-insensitive3) and EIL1 (EIN3-like protein) protein stability is enhanced by ethylene treatment (Guo and Ecker, 2003), but reduced by glc

treatment (Yanagisawa et al. 2003). In general, ABA and glc responsiveness are correlated, whereas ethylene acts antagonistically to these signals at germination and in early seedling growth.

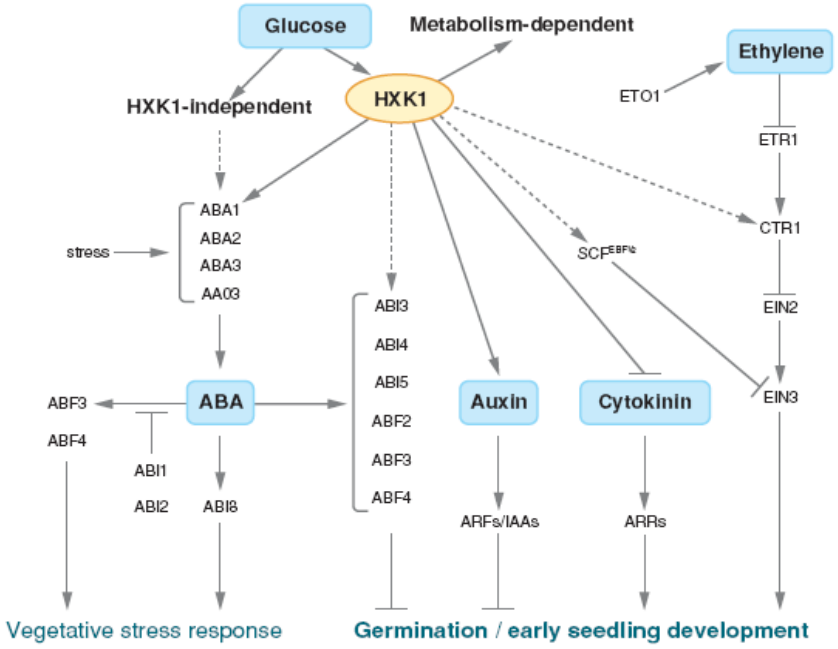

Figure 1.2 A Model for interaction between glc and hormone signaling. Glc signaling interacts with ABA ethylene, auxin and cytokinin signaling to modulate plant growth. Overall, ABA has same responses where as ethylene and auxin show antagonistic responses to glc signaling. [Figure was adapted from Rolland et al. (2006)]

Like ABA and ethylene, gibberellins are also involved in this complex web of sugar signaling. For example glc can repress gibberellin induction of α-amylases in barley embryo (Perata et al. 1997). Site-directed mutagenesis of the 50-bp nucleotide sequence from -172 to -123 of *α-amylase* genes in rice revealed that consensus sequences of G motif (TACGTA) and TATCCA T/C are responsible for its sugar repression

(Toyofoku et al.1998). Lu et al. (1998) identified target sequences in the promoter of a rice α amylase (*αAmy3*) containing the GC box plus, either G box or the TATCCA element, of which TATCCA element is a component of the GA response. Morita et al. (1998) demonstrated that the gibberellin-inducible rice α-amylase gene, *RAmy1A*, is sugar repressed in rice embryos. They also found that gibberellin-response *cis*-elements of GARE (TAACAAA) and pyrimidine box (CCTTTT) were partially involved in the sugar repression. Three MYB factors that bind to the glc and gibberellin response element TATCCA have recently been identified from rice (Lu et al. 2002).

The delayed leaf senescence and defects in cell expansion in *gin2* also suggested a possible connection between glc and cytokinin signaling (Moore et al. 2003) (Fig. 1.2). When supplemented with cytokinin, WT Arabidopsis seedlings could overcome the developmental arrest induced by 6% glc (Moore et al. 2003). Conversely, cytokinin signaling mutants *ahk3* and *cre1* are sugar hypersensitive (Franco-Zorrila et al. 2004). These findings suggest that glc and cytokinin signaling can be antagonistic. However, HXK-dependent glc signaling also interacts with cytokinin signaling as regulatory components of mitosis by promoting the expression of cyclinD2 and cyclinD3, respectively (Riou-Khamlichi et al. 2000). In tobacco BY-2 cells, expression of the D cyclins is upregulated by auxin, but differentially affected by sugars and cytokinins: CycD2:1 is upregulated by hexoses but downregulated by cytokinins, whereas expression of CycD3:1 is governed in the opposite way. CycD3:2 is synergistically upregulated by all three types of signals (Hartig and Bake, 2005), indicating that the glc interaction with cytokinin is tissue dependent.

The phytohormone auxin is a key regulator of plant growth and development. Glc signaling also interacts with auxin signaling. Moore et al. (2003) have reported a decreased sensitivity to auxin by *gin2,* despite seedlings having normal auxin levels (Fig. 1.2). Furthermore, the auxin signaling mutants *axr1, axr2* and *tir1* are insensitive to glc-induced developmental arrest on plates with 6% glc. These observations support an interaction between glc signaling and auxin signaling, downstream of HXK1.

In addition to interaction with phytohormone signaling pathways, there are reports of cross talk between sugar-sensing and various light signals. In the experiments by Thum et al. (2003), light was able to override carbon as a major regulator of *ASN1* (asparagine synthetase) and *GLN2* (glutamine synthetase) in etiolated seedlings. By contrast, carbon potentiates far red induction of *GLN2* and *ASN2* in light-grown plants. In a microarry analysis conducted by Thum et al. (2004), the effects of both light and sugar were examined. The results reveal that the majority of affected genes are co-regulated by both stimuli.

Other than light, there are also reports of the influence of essential mineral nutrients such as nitrogen and phosphorus on carbohydrate metabolism and related gene expression. The effects of nitrogen and a combination of both glc and nitrogen were analyzed by microarray analysis (Price et al. 2004). Interestingly, most of the nitrogen responses seem to require the presence of a carbon source. Karthikeyan et al. (2007) showed that sugar is required for Pi starvation responses, including changes in root architecture and expression of phosphate starvation induced (PSI) genes in Arabidopsis. They further investigated the role of HXK1 and found significant differences in the root

architecture as well as the expression profile of PSI genes between WT and *gin2-1*. All the PSI genes were expressed at a reduced level in *gin2-1*, suggesting the involvement of HXK-dependent glc signaling in Pi starvation.

Hexokinases

HXKs are the family of enzymes that catalyze the transfer of γ-P of ATP to C6 of hexoses. It is now well established that HXK is present in virtually all living organisms (Cardenas et al. 1998). Glc is the preferred substrate of HXKs, but they can also phosphorylate other hexoses to varying degrees (Cardenas et al. 1998). HXKs from different species differ in molecular mass and tissue distribution and often exist as a mixture of isozymes that differ in kinetic properties (Cardenas et al. 1998). The molecular mass of hexokinases from different organisms follows a geometric series 25:50:100.

Bacteria possess the smallest HXKs, approximately 25 kDa. Bacteria have a series of HXKs, each one acting on a specific hexose. In eukaryotes, the molecular mass of HXK ranges between 50-100 kDa. The substrate specificity is often lost and in general non-specific HXKs are characteristic of higher eukaryotes.

HXKs in many species exist as a mixture of isozymes that often differ in kinetic characteristics. Multiple HXKs were first demonstrated in yeast (Trayser et al. 1961). The yeast genome encodes for two homologous HXKs PI and PII. Both the HXKs in yeast have a molecular mass of 50 kDa and can form dimers (Cardenas et al. 1998).

HXK PI and PII can phosphorylate both glc and fructose (Lobo and Maitra, 1977), but HXK PII is the predominant form in yeast grown on glc as carbon source (Gancedo et al. 1977).

Based on ion exchange chromatography (Gonzalez et al. 1964) or electrophoresis (Katzen and Schimke, 1965) of the extracts of various mammalian tissues, HXKs can be classified into four different isozymes (Type I, II, III and IV). The type I-III are 100 kDa molecules and where as Type IV HXK in a 50 kDa protein. More recently a fifth HXK (HKDC1) has been identified and likely encodes a 100 kDa protein (Irwin and Huanran, 2008).

The existence of HXKs in plants was first demonstrated by Saltman (1953) working with wheat germ. Like most eukaryotic HXKs, plant HXKs can phosphorylate multiple hexoses including D-glc, D-fructose and D-mannose, and are often encoded by a modestly large gene family (Claeyssen and Rivoal, 2007). The analysis of available genome sequencing data indicates that HXK is encoded by a multigene family in evolutionarily diverse plant species (Virnig and Moore, unpublished data). Among the well studied plant species, the rice genome encodes at least ten HXKs (Cho et al. 2006), Arabidopsis six HXKs (Rolland et al. 2002), and tomato has at least four HXKs (Kandel-Kfir et al. 2006).

Among different plant HXKs, the K_m for glc is low and varies between 15-150 μM. In contrast, the K_m for fructose is always in the mM range (Renz and Stitt, 1993, Martinez and Randall, 1998; Higgins and Easterby 1974; Galina et al. 1995; Giese et al. 2005). The K_m-ATP is 50-560 μM, however some forms can use UTP (Rentz et al 1993;

Schnarrenberger, 1990). Plant HXKs differ from mammalian HXKs with respect to their sensitivities to glc-6P. Mammalian HK I, II, III are extremely sensitive to inhibition by glc-6P. The sensitivity of plant HXKs varies with isoforms. For example, in potato tubers the activity of HXK1 is inhibited by G6P where as HXK2 is insensitive to G6P inhibition (Rentz and Stitt, 1993). In general, most plant HXKs are insensitive to glc-6P, but readily inhibited by ADP (Claeyssen and Rivoal, 2007).

One of the more intriguing aspects of HXK in different organisms is the different intracellular locations observed for diverse family members. In yeast, HXK2 is reported to have dual targeting to both the nucleus and cytosol (Randez-Gil et al. 1998). In plants, HXK proteins are reported to occur in the cytosol, mitochondria, plastids, nuclei, and Golgi and plasma membrane (Miernyk and Dennis, 1983; Schnarrenberger, 1990; da-Silva et al. 2001; Yanasigawa et al. 2003). The N-terminus of several plant HXKs has been implicated in their targeting to specific cell compartments (Wiese et al. 1999; Olsson et al. 2003; Giese et al. 2005; Claeyssen et al. 2006). The HXKs have been classified as either type A or type B, based on the N-terminal sequence. Type A HXKs are predicted to be targeted to mitochondria where as type B HXKs are targeted to the secretory pathway (Claeyssen and Rivoal, 2007; Olsson et al. 2003). Some of these the bioinformatics predictions are supported by experimental evidence.

In pea (*Pisum sativum*) leaves, most of the HXK is associated with mitochondria and facilitates respiration (Dry et al. 1983). In spinach (*Spinacia oleracea*) leaves, SoHXK1 which was initially shown to be associated with the external plastid envelope (Wiese et al. 1999) was later shown to be a mitochondria associated HXK (Damari-

Weissler et al. 2007). In moss (*Physcomitrella patens),* PpHXK1 is localized to the chloroplast stroma (Olsson et al. 2003). Rice (*Oryza sativa*) has more than one cytosolic HXK (Cho et al. 2006). Available evidence suggests that plastid localized HXKs might occur only in the stroma and possibly stromules (Kandel-Kfir et al. 2006), and might occur most abundantly in certain sink tissues (Giese et al. 2005). It has also been hypothesized that the sub-cellular location and kinetic properties of each HXK isoform relates to its function.

HXK is best known as a glycolytic gateway enzyme. But apart from its central role in metabolism it has been assigned several regulatory functions. The role of HXK as a glc sensor has already been discussed. As another function, the binding of HXK to mitochondria blocks apoptosis in cancer cells (Pastorino et al. 2002; Majewski et al. 2004) and it has recently been shown that plant mitochondrial HXKs also regulate programmed cell death (Kim et al. 2006). Since catalytic and regulatory functions of AtHXK1 are separable from each other (Moore et al. 2003), HXK1 is therefore a moonlighting protein with possibly novel functions yet to be discovered (Moore, 2004).

Non catalytic HXKs

There are reports of non catalytic HXKs which have been assigned regulatory functions. There are two hexokinase-like (HKL) proteins in *S. cerevisiae* (Bernardo et al. 2007), one of which, EMI2, is required for induction of a meiosis-specific transcription factor (Daniel 2005). In *Aspergillus nidulans, HxkC* and *HxkD* [earlier reported as *xprF*

by Katz et al. (2000)] encode for non catalytic HXKs and are localized to the nucleus and mitochondria respectively. HxkD has a role in carbon catabolic repression of extracellular proteases (Katz et al. 2000) and *hxkC*Δ exhibits a growth phenotype similar to *hxkD*Δ (Bernardo et al. 2007). HxkC is a negative regulator of the putative transcription factor, XprG, which is involved in the response to nutrient limitation and may have a role in the TOR-signaling pathway (Katz et al. 2006). Kulkarni et al. (2002) isolated two HKL proteins from *Drosophila melanogaster* , namely DHK-465 (Drosophila HKL with 465 aa residues) and DHK-453 (Drosophila HKL protein with 453 aa residues). DHK-453 shows conservation of all the residues significant for substrate binding and catalysis and is proposed to have a role in cellular functions other than catalysis. It is likely that these non catalytic HXKs have evolved from their catalytic counterparts by acquisition of a moonlighting function (Brenardo et al. 2007). This raises the question of the possible functions of individual HXK isoforms.

The Arabidopsis genome encodes six HXK isoforms (Karve et al. 2008). The goal of my research project is to understand the function of these putative HXKs. In particular I am interested in understanding the role of the HXK isoform designated as AtHKL1 (AGI: At1g50460). HKL1 has been found associated with mitochondria in the proteome analysis conducted by Giege et al. (2003). Analysis of Arabidopsis EST databases by Claeyssen and Rivioal (2007), has implicated its role along with that of two other HXKs in stress responses. In an attempt to characterize the function of this HXK gene family member, the amino acid sequences, expression features and the glucose phosphoryation activities of all six HXK gene family members were first characterized.

HKL1 is ~50% identical to HXK but did not phosphorylate glc or fructose. However, HKL1 is expressed in all the tissues where HXK1 is expressed, and like HXK1, is localized to mitochondria. To test whether HKL1 might have a role in regulation of plant growth, transgenic Arabidopsis with altered HKL1 expression were characterized. In addition, HKL1 tissue expression and regulation was studied in transgenic *HKL1* promoter-*GUS* lines. The phenotypes of HKL1 transgenic and mutant lines indicated a crucial role of HKL1 in plant growth that is dependent on HXK1. The phenotypes of HKL1 transgenic and mutant lines and the promoter regulation suggested a role for HKL1 in mediating cross-talk with hormone signaling pathways. The involvement of HKL1 in cross-talk was further examined by using a candidate gene approach and by characterizing additional phenotypes of HKL1 transgenic and mutant lines. The results of gene expression analysis and phenotypic assays indicated that HKL1 has a role in mediating crosstalk with ethylene signaling. The results of co-immunoprecipitation assays show that HKL1 and HXK1 can physically interact. This interaction might be required for observed HKL1 phenotypes.

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CHAPTER TWO

EXPRESSION AND EVOLUTIONARY FEATURES OF THE HEXOKINASE GENE FAMILY IN ARABIDOPSIS

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Abstract

Arabidopsis hexokinase1 (HXK1) is a moonlighting protein that has separable functions in glucose signaling and in glucose metabolism. In this study, we have characterized expression features and glucose phosphorylation activities of the six HXK gene family members in *Arabidopsis thaliana*. Three of the genes encode catalytically active proteins, including a stromal-localized HXK3 protein that is expressed mostly in sink organs. We also show that three of the genes encode hexokinase-like (HKL) proteins, which are about 50% identical to AtHXK1, but do not phosphorylate glucose or fructose. Expression studies indicate that both HKL1 and HKL2 transcripts occur in most, if not all, plant tissues and that both proteins are targeted within cells to mitochondria. The HKL1 and HKL2 proteins have 6-10 amino acid insertions/deletions

(indels) at the adenosine binding domain. In contrast, HKL3 transcript was detected only in flowers, the protein lacks the noted indels, and the protein has many other amino acid changes that might compromise its ability even to bind glucose or ATP. Activity measurements of HXKs modified by site-directed mutagenesis suggest that the lack of catalytic activities in the HKL proteins might be attributed to any of numerous existing changes. Sliding windows analyses of coding sequences in *A. thaliana* and *A. lyrata* ssp. *lyrata* revealed a differential accumulation of nonsynonymous changes within exon 8 of both HKL1 and HXK3 orthologs. We further discuss the possibility that the non-catalytic HKL proteins have regulatory functions instead of catalytic functions.

Introduction

Sugars are the primary currency in the metabolic economy of most cellular life. Contemporary research has revealed remarkable interconnections between the cellular and molecular processes that govern production or acquisition of sugars and their efficient utilization. In plants, sugars regulate plant growth and development by interacting with many different control processes, including ones with meristematic functions (Smeekens 2000; Francis and Halford 2006; Rolland et al. 2006). For example, in Arabidopsis both glucose and sucrose modulate the expression of nucleolin, a multi-function regulator of ribosome synthesis (Kojima et al. 2007). An increased amount of nucleolin was suggested to be a key component in the process by which sugars can enhance meristematic cell division activity. Interestingly, the targeted expression of cell wall invertase in apical meristems of Arabidopsis was shown to influence the developmental transition to flowering and ultimately to increase seed yield (Heyer et al. 2004).

Short-term treatments of Arabidopsis seedlings with glucose or sucrose have been shown to affect the expression of about 1000 to 1700 transcripts, depending on experimental conditions (Price et al. 2004; Osuna et al. 2007). Sugars can influence plant gene expression both through general metabolic effects and as signal molecules that can directly interact with sensor/transducer proteins (Sheen et al. 1999; Xiao et al. 2000). Arabidopsis hexokinase1 (HXK1) is perhaps the best characterized glucose signaling protein. The isolation and characterization of a null mutant of AtHXK1, *gin2-1*, revealed

associated phenotypes including reduced shoot and root growth, reduced leaf expansion, increased apical dominance, delayed flowering and senescence, decreased auxin sensitivity, increased cytokinin sensitivity, and changes in transcript levels of several target genes (Moore et al. 2003). Furthermore, complementation of these phenotypes by transformation of *gin2-1* with a catalytically compromised HXK1 protein (S177A) demonstrated that the HXK metabolic function can be uncoupled from its signaling and related growth promoting functions. AtHXK1 is, therefore, a moonlighting protein (Moore 2004). The multi-function nature of AtHXK1 can be viewed as a novel cellular solution to integrate glucose metabolism with a separable glucose signal transduction process.

The biochemical basis for AtHXK1 function as a moonlighting protein has not been established. Analysis of crystal structures of other moonlighting proteins has shown that one mechanism for acquiring a moonlighting function is the development of distinct surface features that mediate protein-protein interactions (Jeffrey 2004). While a crystal structure of AtHXK1 is not available, much might be learned by a close inspection of its evolutionary heritage and of existing structural homologs. Bork et al. (1992) suggested that a prokaryotic, dimeric ancestral ATPase has evolved through diverse processes into structurally related families of actin, hexokinase, and heat shock protein 70. The relatively sugar non-specific, but ATP-dependent hexokinases are characteristic of higher Eukarya and are thought to have arisen from a common ~50 kDa ancestral protein (Cárdenas et al. 1998). As far as is known, hexokinases are present in a given eukaryote as a multi-gene family. Surprisingly, within some of these gene families, individual

members can have specialized non-catalytic, regulatory functions. For example, there are 2 described hexokinase-like (HKL) proteins in *Aspergillus nidulans*. They both lack catalytic activity, but they are negative regulators for secretion of an extracellular protease in response to carbon starvation (Bernardo et al. 2007). In this case, sequence analysis suggested that specific amino acid changes relative to the canonical sequence are responsible for the lack of catalytic activity. It remains to be determined whether specialized regulatory HXKs occur within plant HXK gene families.

Phylogenetic analyses of a variety of plant HXKs indicate that these occur largely in 2 groups, ones with plastid signal peptides (Type A) and ones with N-terminal membrane anchors (Type B; Olsson et al. 2003). Direct experimental evidence for stromal-localized HXKs have been reported from moss (Olsson et al. 2003), tobacco (Giese et al. 2005), rice (Cho et al. 2006a), and tomato (Kandel-Kfir et al. 2006). Plastidic NtHXK2 is expressed mostly in certain starch-containing sink tissues, while plastidic LeHXK4 occurs in both source and sink organs, including non-starch containing fruits. Membrane-bound HXKs occur largely, but not exclusively, associated with mitochondria (Kandel-Kfir et al. 2006 and references therein; Damari-Weissler et al. 2006). AtHXK1 is predominantly associated with mitochondria, but also can occur in the nucleus (Cho et al. 2006b; Balasubramanian et al. 2007); both forms can modulate gene expression. Whether the nuclear form of AtHXK1 maintains its membrane anchor is not clear. Rice and maize have one or more cytosolic HXKs (da Silva et al. 2001; Cho et al. 2006a), though these forms might occur only in monocots (Damari-Weissler et al. 2006). A number of different possible metabolic roles of HXKs have been recently described

(Claeyssen and Rivoal 2007). Mitochondrial HXKs are thought to have preferred access to ATP produced in respiration for consumption by active metabolite fluxes through sucrose cycling, glycolysis, and sugar nucleotide syntheses (Rontein et al. 2002; Graham et al. 2007).

Among the better examined plant HXK families, rice has at least nine expressed HXKs (Cho et al. 2006a), tomato at least four HXKs (Kandel-Kfir et al. 2006), and Arabidopsis likely six HXKs (Rolland et al. 2002; Claeyssen and Rivoal 2007). We are interested in the function of Arabidopsis HXKs in organismal space. In this study, we describe their gene structures, their tissue and sub-cellular expressions, and we show that three of the six family members lack catalytic activity. We then did a detailed amino acid sequence analysis to identity key amino acid differences and to test a number of possible mechanisms by which catalysis might be compromised. The presence of non-catalytic HKL proteins in plants raises intriguing questions regarding their evolution and function. A comparison of HXK family coding sequences from *A. thaliana* and *Arabidopsis lyrata* ssp. *lyrata* (hereafter *A. lyrata*) allowed us to identify regions of some gene orthologs that are undergoing possible differential selection.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heyn. Ecotype Columbia (Columbia-0) were

obtained from Arabidopsis Biological Resource Center (Ohio State University). Seeds of maize were purchased (line FR922 X FR967, Seed Genetics, Inc. Lafayette, IN) and dark-grown for 9 days, followed by overnight greening (Jang and Sheen 1994). For most experiments, Arabidopsis was grown in soil in a growth chamber (Balasubramanian et al. 2007), except for collecting root tissue from plants grown by hydroponics (Tocquin et al. 2003). Leaf tissue from *A. lyrata* was kindly provided by Dr. Amy Lawton-Rauh.

Cloning and plasmid constructs

AtHXK1 was previously cloned using *BamH1* and *Stu1* restriction sites into the HBT plant expression vector (Kovtun et al. 1998) followed either with a C-terminal double hemagglutinin (HA) tag (Moore et al. 2003) or with a C-terminal green fluorescent protein (GFP) fusion (Balsubramanian et al. 2007). Leaf or seedling cDNA libraries (see below) were used as template for PCR amplification of AtHXK2 (At2g19860, 5'-CGG GAT CCC GAT GGG TAA AGT GGC AGT TGC AAC G, 5'-AAA AGG CCT ACT TGT TTC AGA GTC ATC TTC), AtHXK3 (At1g47840, 5'-CGG GAT CCC G AT GTC ACT CAT GTT TTC TTC CCC TGT C, 5'- AAA AGG CCT GTA AAT GGA GTT AGT GGC CGC C), AtHKL1 (At1g50460, 5'-CGG GAT CCC GAT GGG GAA AGT GGC GGT TGC G, 5'-AAA AGG CCT TGA CTG TAA AGA GGC AAC GAG GAG), AtHKL2 (At3g20040, 5'-CGG GAT CCA TGG GGA AGG TTT TGG TGA TGT TG, 5'-AAA AGG CCT TAC GGA TGG TAT TGT TTG AAC AC), and AtHKL3 (At4g37840, 5'-TGC CAT GGC ATG ACC AGG AAA GAG

GTG GTT C, 5'-GAA GGC CTC TTG CTT TCA GAA TCT TGA TGA). PCR products were then ligated into an HBT vector with the double HA tag, using the *BamHI/StuI* restriction sites for most constructs or available *NcoI/StuI* sites for AtHKL3. All clones were validated by direct sequencing of plasmid DNA and by predicted sizes of the expressed proteins. The coding sequences were then sub-cloned into the same vector, but with a GFP tag.

Site-directed changes, insertions, and deletions of native sequences were all made by Quick Change (Stratagene). For AtHXK1, the target amino acid changes and primers were as follows: N106Y (5'-GGA CCT AGG GGG GAC ATA CTT CCG TGT CAT GCG TG, 5'-CA CGC ATG ACA CGG AAG TAT GTC CCC CCT AGG TCC), G173A (5'-GGT AGA CAG AGG GAA TTA GCC TTC ACT TTC TCG TTT CC, 5'-GG AAA CGA GAA AGT GAA GGC TAA TTC CCT CTG TCT ACC), L251F (5'-G GAT GTT GTT GCT GTT ATT TTC GGC ACT GGG ACA AAC G, 5'-C GTT TGT CCC AGT GCC GAA AAT AAC AGC AAC AAC ATC), C159E (5'- G AAG TTT GTC GCT ACA GAA GAG GAA GAC TTT CAT CTT CC, 5'-GG AAG ATG AAA GTC TTC CTC TTC TGT AGC GAC AAA CTT C), and insert ^{428}GITSGRSRSE^{437} (5'-CTG GGA AGA GAT ACT ACT AAA GGA ATC ACC AGC GGA AGA TCT AGA AGC GAG GAC GAG GAG GTG CAG AAA TCG G, 5'-C CGA TTT CTG CAC CTC CTC GTC CTC GCT TCT AGA TCT TCC GCT GGT GAT TCC TTT AGT AGT ATC TCT TCC CAG). For AtHKL1, amino acids ^{425}GITSGRSRSE^{434} were deleted similarly (5'-GAT AGG CCG AGA TGG AAG CAG AAG TGA AAT CCA AAT G, 5'-CAT TTG GAT

TTC ACT TCT GCT TCC ATC TCG GCC TAT C). All mutations were verified by DNA sequencing.

Analysis of mRNA expression by RT-PCR

Total RNA was prepared using the RNeasy kit (Qiagen) from 100 mg of corresponding plant tissue from *A. thaliana*. Root tissue was collected from plants grown in hydroponics, while all other tissues were from soil grown plants. One μg of total RNA was converted to cDNA using the Protoscript II RT-PCR kit according to the manufacturer's instructions (New England BioLabs). PCR primer sequences were generated using the AtRTPrimer public database (Han and Kim 2006). Primers in all cases span one or more introns: AtHXK1 (5'-TGC TGC TTT CTT TGG CGA TAC AGT, 5'-AAA ATG GCG CTC TTT GGG TAG GTT; expected size = 505 bp), AtHXK2 (5'- ACA AAT GCA GCC TAT GTC GAA CGT G, 5'-TGT TCG GGG TCC TTA TGA TGA ATG G; expected size = 316 bp), AtHXK3 (5'- TCT CGA CCA CGC TCC AAT TAC ATC, 5'-AAT CAC ACC GAC CAT CAC ATC CTC; expected size = 702 bp), AtHKL1 (5'- GTT GGA GCC TTG TCG CTT GGA TAT T, 5'-CCT GCT CTT CGT GTA ACC ACA TCG; expected size = 521 bp), AtHKL2 (5'- CCC AGT CAA GCA GAC ATC CAT CTC A; 5'-TCG CCC AGA TAC ATC CCT CCT ATC A, expected size = 441 bp), and AtHKL3, (5'- TGG AAA CAC ACG GTC TGA AAA TTC G; 5'-TCA TCA CCA AGC ATT TCC CAA ACG, expected size = 736 bp). As a control for amount of tissue template, we routinely used AtUBQ5 (At3g62250, 5'-GTG GTG CTA

AGA AGA GGA AGA, 5'-TCA AGC TTC AAC TCC TTC TTT; expected size = 254 bp). For PCR, we used 0.5-0.8 μl of cDNA in 10 μl reactions to first balance UBQ expression for the set of tissue cDNAs, with corresponding tissue template concentrations used thereafter for PCR reactions of varying cycle numbers for each product.

Protoplast transient expression and ^{35}S-labeling

Protoplasts were isolated from Arabidopsis leaves (Hwang and Sheen 1990) or greening maize leaves (Jang and Sheen 1994). These were transfected using the polyethylene glycol 4000 (Fluka) protocol (Yoo et al. 2007) and 6-12 μg of cesium chloride-purified plasmid DNA. In some experiments, newly synthesized proteins were labeled with [^{35}S]Met (Perkin Elmer) for 8 h, then collected from lysed protoplasts onto protein A agarose beads using anti-HA antibodies (Roche) as detailed previously (Balasubramanian et al. 2007). Proteins were solubilized in 2X SDS treatment buffer, electrophoresed on 10% SDS gels, and visualized by fluorography.

Enzyme activity assays

HXK was assayed largely as described by Doehlert (1989) in a medium containing 50 mM Bicine-KOH pH 8.5, 5 mM $MgCl_2$, 2.5 mM ATP, 1 mM NAD, 15 mM KCl, 2 units of glucose 6-phosphate dehydrogenase (Sigma G8404), and either 2 mM glucose or 100 mM fructose. The increase in A_{340} was monitored over a 30 min

interval and rates then calculated accordingly. Transfected protoplasts from greening maize leaves were used in either of 2 ways as a source of possible enzyme activity. First, frozen protoplast pellets were lysed by vortexing in a standard leaf extraction buffer containing 50 mM Hepes-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 10% glycerol, 0.1% Triton X-100, and 1X protease inhibitor cocktail (Roche). This extract was then assayed directly. Second, HA-tagged proteins were isolated as described above. Thrice-washed beads were resuspended in enzyme activity assay buffer (minus sugar), transferred to cuvettes, and rates then measured after adding glucose or fructose. Immobilized protein had lower enzyme activity, but the recovery rates were very consistent (20 ± 2 %). Included protoplast controls that received non-coding plasmid DNA had little or no background due to endogenous HXK activity that was initially present in the maize protoplasts.

Analysis of HXK family sequences in A. thaliana and A. lyrata

We first queried the NCBI database using BLAST and specifying the *A. lyrata* WGS first draft sequence database, using the coding sequence for each of the HXK family member genes from *A. thaliana* as the query sequences. From this, we identified the homologous exons, introns, and splice sites for each of the six genes from the genome project of *A. lyrata*. To correct potential sequence errors we identified within exon 6 of *AlHXK3*, we directly sequenced the PCR product from first strand cDNA synthesis of the

corresponding transcript. Otherwise, the available sequence information was robust with multiple reads and the splice sites were highly conserved.

All nucleotide and amino acid sequences were aligned manually using BioEdit (Hall 1999) and exported as Nexus or FASTA files. Phylogenetic analyses of the HXK and HKL loci were conducted using amino acid alignments in MEGA build 4024 (Tamura et al. 2007). Phylogenetic trees were estimated using the neighbor-joining method, 1000 bootstrap replicates, and the Dayhoff substitution model. Codon usage bias was examined using DnaSP v4.20.2 (Rozas et al. 2003), then calculated as effective number of codons (Wright 1990) and by the codon bias index (Morton 1993). Nucleic acid sequence diversity was estimated using DnaSP v4.20.2 (Rozas et al. 2003) to calculate ω ratios (number of nonsynonymous substitutions per nonsynonymous site/number of synonymous substitutions per synonymous site, K_A/K_S), following Nei and Gojobori (1986), and sliding window analysis of K_A/K_S ratios. All nucleotide sequence based analyses used paired alignments of coding sequences of *A. thaliana* and *A. lyrata* on a gene by gene basis.

Results

Gene structure of Arabidopsis HXK family

The *Arabidopsis thaliana* genome potentially encodes six HXK related proteins (TAIR). By pair-wise Blast searches, these predicted proteins range from 45-85%

identical to AtHXK1 (Table 2.1). As detailed below, we have designated these as HXK proteins or as hexokinase-like (HKL) proteins based, respectively, on whether they have apparent glucose phosphorylation activity or whether they lack catalytic activity. For reference, AtHXK1 is 36% identical to yeast HXK2 and about 70% identical to rice HXK2 and to tomato HXK1.

Table 2.1 Selected properties predicted for HXK family proteins from *A. thaliana*

	% Identity	Transmembrane domain	Transit peptide
HXK1	100	8–24	No
HXK2	85	8–24	No
HXK3	53	No	1–34
HKL1	54	8–24	No
HKL2	49	4–20	No
HKL3	45	6–22	No

Amino acid sequences were subjected to pair-wise Blast anaylsis, and PSORT (http://psort.nibb.ac.jp/) and Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) targeting analyses

Phylogenetic analysis of the AtHXKs reveals several interesting features (Fig.2.1 A). Bootstrap replicate values suggest that the gene pairs HXK1 and HXK2, and HKL1 and HKL2 are more closely related to each other than they are to other genes in the family. When analyzed with the 10 reported rice HXK family members, AtHKL1 and AtHKL2 form a related sub-group with OsHXK3 and OsHXK10 (Cho et al. 2006a). Within *A. thaliana*, HKL3 forms a distinct and not closely related sub-group. AtHKL3

also was reported not to form any closely related phylogenetic sub-groups with rice HXKs (Cho et al. 2006a). The genome structure of the AtHXKs shows that most have 9

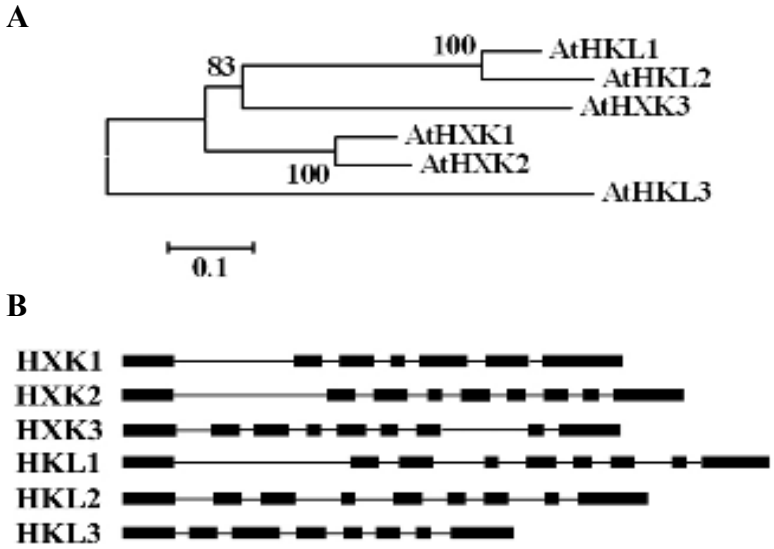

Figure 2.1 (A) Phylogenetic relationships of predicted Arabidopsis HXK family proteins. As described below, proteins with detectable catalytic activity are designated as HXK proteins. Those without detectable catalytic activity are designated as HKL proteins. HXK1 = At4g29130; HXK2 = At2g19860; HXK3 = At1g47840; HKL1 = At1g50460; HKL2 = At3g20040; HKL3 = At4g37840. Phylogenetic reconstruction was done using MEGA4 (Tamura et al. 2007). The *scale bar* = the frequency of amino acid substitutions per unit length. (B) Illustrations of gene structures were determined from genomic sequence data (NCBI, TAIR). Exons are indicated by rectangles, introns by lines. The lengths of both are proportional to the number of nucleotides that are present.

exons, except for HXK1 which has 7 and HKL3 which has 8 (Fig. 2.1 B). Most rice HXKs also have 9 exons (Cho et al. 2006a). The intron structures of AtHXKs vary among the different family members. Intron1 in HXK1, HXK2, and HKL1 is relatively long, ranging from 625-804 nucleotides, while intron1 in HXK3, HKL2, and HKL3 is shorter, ranging from 72-183 nucleotides. HKL3 has short introns throughout the gene, averaging 81 nucleotides.

Organ expression of AtHXKs mRNAs and cellular expression of AtHXKs proteins

We examined by semi-quantitative RT-PCR the organ expression of transcripts for the *A. thaliana* HXK gene family (Fig. 2.2). HXK1 transcript was abundant in all organs examined. HXK2 transcript was expressed to a relatively similar extent in leaves, but less so in sink tissues.

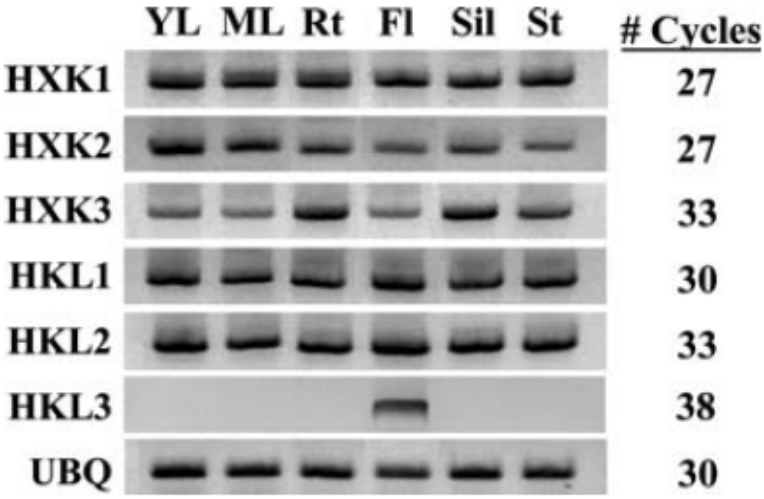

Figure 2.2 Organ expression of Arabidopsis HXK family genes determined by RT-PCR. Ubiquitin5 (UBQ) was amplified as a control for amount of template. Product sizes ranged from 254-702 bp. Additional PCR reactions were run with all primer pairs at increased and decreased cycle numbers to establish reported conditions (data not shown). YL = young leaves; ML = mature leaves; Rt = root; Fl = flower; Sil = silique; St = stem.

HXK3 mRNA was relatively more abundant in sink tissues such as roots and siliques, but the increased number of PCR cycles used for its amplification indicates that it likely is not as abundant as is the HXK1 transcript. HKL1 and HKL2 transcripts were not expressed as highly as HXK1 mRNA, but it is noteworthy that all 3 of these transcripts were expressed at relatively similar levels in all organs examined. In contrast, HKL3 mRNA was detected only in flowers and at relatively much lower amounts.

Proteomic analyses have indicated that most of the HXK family members are associated with mitochondria (Heazlewood et al. 2004). Sequence analysis supports this evidence as well, indicating that HXK1, HXK2, HKL1, HKL2, and HKL3 all have predicted N-terminal transmembrane peptides that target to the mitochondria (Table 2.1). These possible targeting peptides are located upstream from the conserved large domain of the HXKs (Appendix A). However, HXK3 has a putative N-terminal transit peptide, which indicates that it might be expressed in plastids. We examined the subcellular localization of the AtHXK proteins by cloning these as C-terminal GFP fusions, followed by transient expression of their cDNAs in leaf protoplasts, and subsequent imaging of expressed fluorescence (Fig.2.3 A-I). As shown previously (Balasubramanian et al. 2007; Damari-Weissler et al. 2007), HXK1-GFP fluorescence occurred under these conditions only at the mitochondria. HXK2, HKL1, HKL2, and HKL3-GFP fluorescence also was observed only at the mitochondria. Expression of these proteins in protoplasts does apparently cause the mitochondria to aggregate as noted before with HXK1, but co-staining with MitoTracker dyes always showed their GFP fluorescence to be mitochondrial localized (e.g., compare fluorescence patterns in Fig.2.3 D and E). In contrast, HXK3-GFP fluorescence is expressed in chloroplasts (Fig. 2.3 G, H). In the presented image, much of the GFP fluorescence occurs inside the chloroplasts, but some also is located on the outer surface. The latter might be due to protein accumulation at sites of import. Internal accumulation of GFP was shown by using a specific band pass filter that excludes chlorophyll fluorescence. In contrast to these fluorescence patterns, transfected yeast HXK2-GFP is expressed exclusively in the cytosol of leaf protoplasts

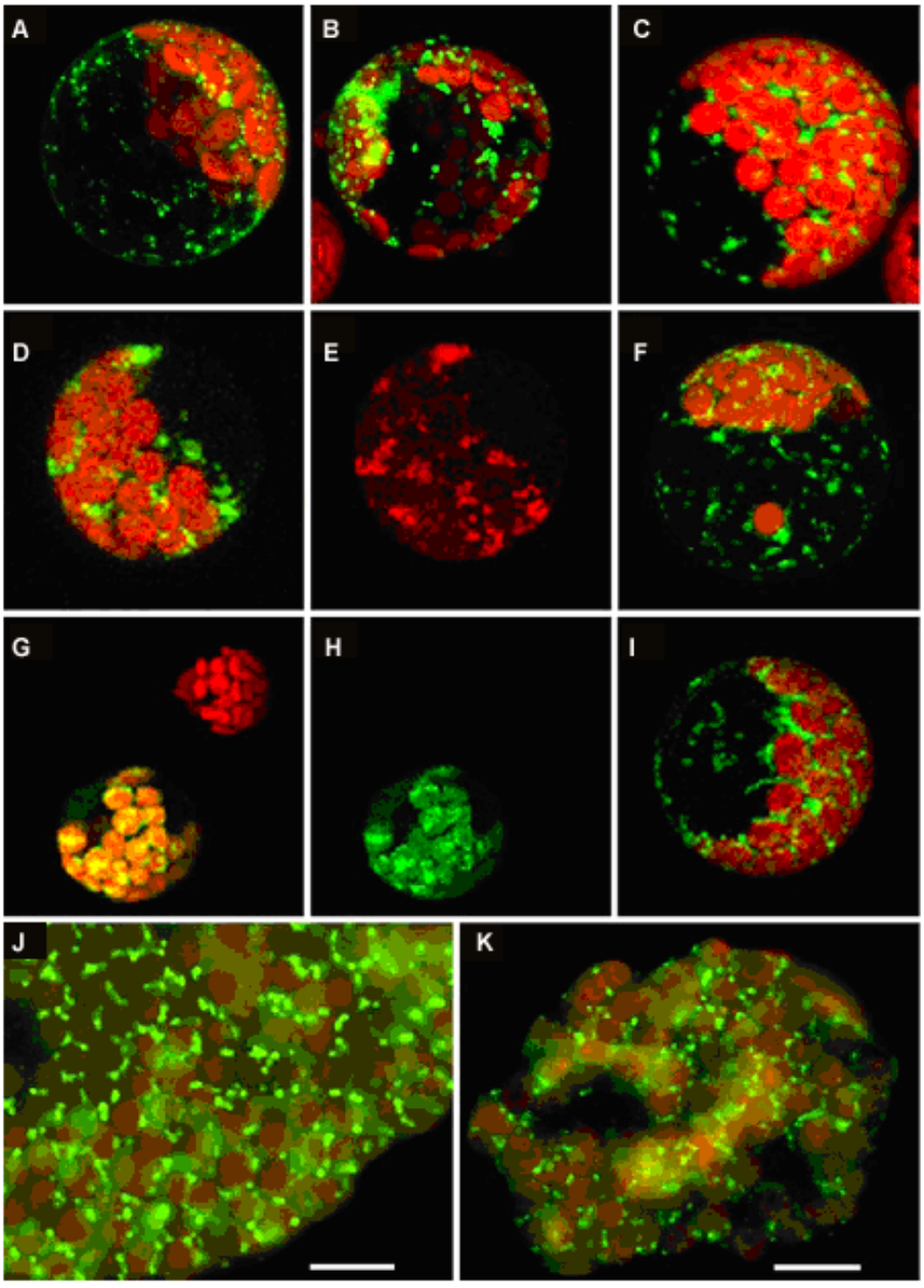

Figure2.3 Sub-cellular expression of Arabidopsis HXK family proteins. (A-I) C-terminal GFP fusion proteins were cloned and expressed in leaf protoplasts, then imaged with a scanning laser confocal microscope. (A) Porin-GFP, a mitochondrial marker. (B) HXK1-GFP. (C) HXK2-GFP. (D) HKL1-GFP. (E) Protoplast in (D) showing selective staining with MitoTracker Red (pseudocolored *red*). (F) HKL2-

GFP. (G) HXK3-GFP, showing merged GFP and chlorophyll fluorescence in lower protoplast (*yellow-orange*). The upper protoplast did not express GFP (*red* only). (H) Protoplasts in (G) showing only the GFP channel. (I) HKL3-GFP. (J-K) Cellular immunolocalization of HXK in leaf mesophyll cells of young seedlings. Following cryofixation and freeze substitution, cells were labeled with anti-HXK1 polyclonal antibody and then with secondary antibody conjugated to FITC. Leaf cells were observed by fluorescence microscopy. Images show a single optical section. *Scale bars* = 10 μm. (J) Leaf cells of Ler WT seedlings. (K) Leaf cells of *gin2-1* seedlings, an HXK1-null mutant.

(data not shown). This localization is consistent with the absence of an N-terminal membrane anchor in ScHXK2.

As a complementary approach to demonstrate the subcellular localization of AtHXKs, we have immunostained seedling leaves after their cryofixation and acetone freeze substitution. First, samples were prepared from WT Ler. These were incubated with a polyclonal antibody to AtHXK1, then a FITC-conjugated secondary antibody, and were subsequently viewed by epifluorescence (Fig. 2.3 J). As noted previously with WT cells (Balasubramanian et al. 2007), we observed HXK1 antigen in numerous elongated shapes which appear to be mitochondria and that were often located close to chloroplasts. This staining likely is due to some combination of HXK1, HXK2, HKL1, and/or HKL2 proteins. As a comparison, we next observed HXK antigen in leaves of *gin2-1* (Fig.2.3 K), a HXK1-null mutant (Moore et al. 2003). We observed a readily detectable, but much reduced level of fluorescence, again associated with elongated, apparent mitochondrial structures. FITC fluorescence was not observed in the cytoplasm or elsewhere in either *gin2-1* or Ler mesophyll cells. Chloroplasts in these cells, however, were not labeled by antibody, perhaps due to the HXK3 protein being present in this tissue only at very low levels, if at all.

Protein sequence analyses of AtHXKs

The HXK tertiary structure is one of the better studied proteins from mammals and yeast. HXK proteins contain a large and a small domain. The sugar binding site is largely located in the small domain, with 4 additional peptide segments (Loops 1-4) that are induced to move upon binding the sugar ligand. These loops both complete the sugar binding sites as well as "pre-form" the nucleotide binding site (Kuser et al. 2000). In fact, analysis of HXK sequences from mammalian, yeast, and Arabidopsis (AtHXK1) using crystal structures, sequence elements, and/or space filling models indicate that most of the conserved amino acid residues occur at the cleft of the 2 domains and form the glucose and ATP binding sites (Kuser et al. 2000). In order to better associate structural differences of AtHXKs with their possible functional differences, we have aligned and analyzed their 1^{o} amino acid sequences (Fig. 2.4). Homologous regions and residues in AtHXKs were assigned based on reported detailed analyses of HXK2 from *Saccharomyces cerevisiae* (Bork et al. 1990; Kuser et al. 2000). By inspection, HXK1, HXK2, and HXK3 were much more similar to each other in the longer motifs designated phosphate 1, connect 1, phosphate 2, connect 2, and adenosine, than were HKL1, HKL2, and HKL3. The relative divergence of HKL3 is particularly pronounced in the phosphate 1 , sugar, and connect 1 motifs. Most noticeably, both HKL1 and HKL2 have an indel

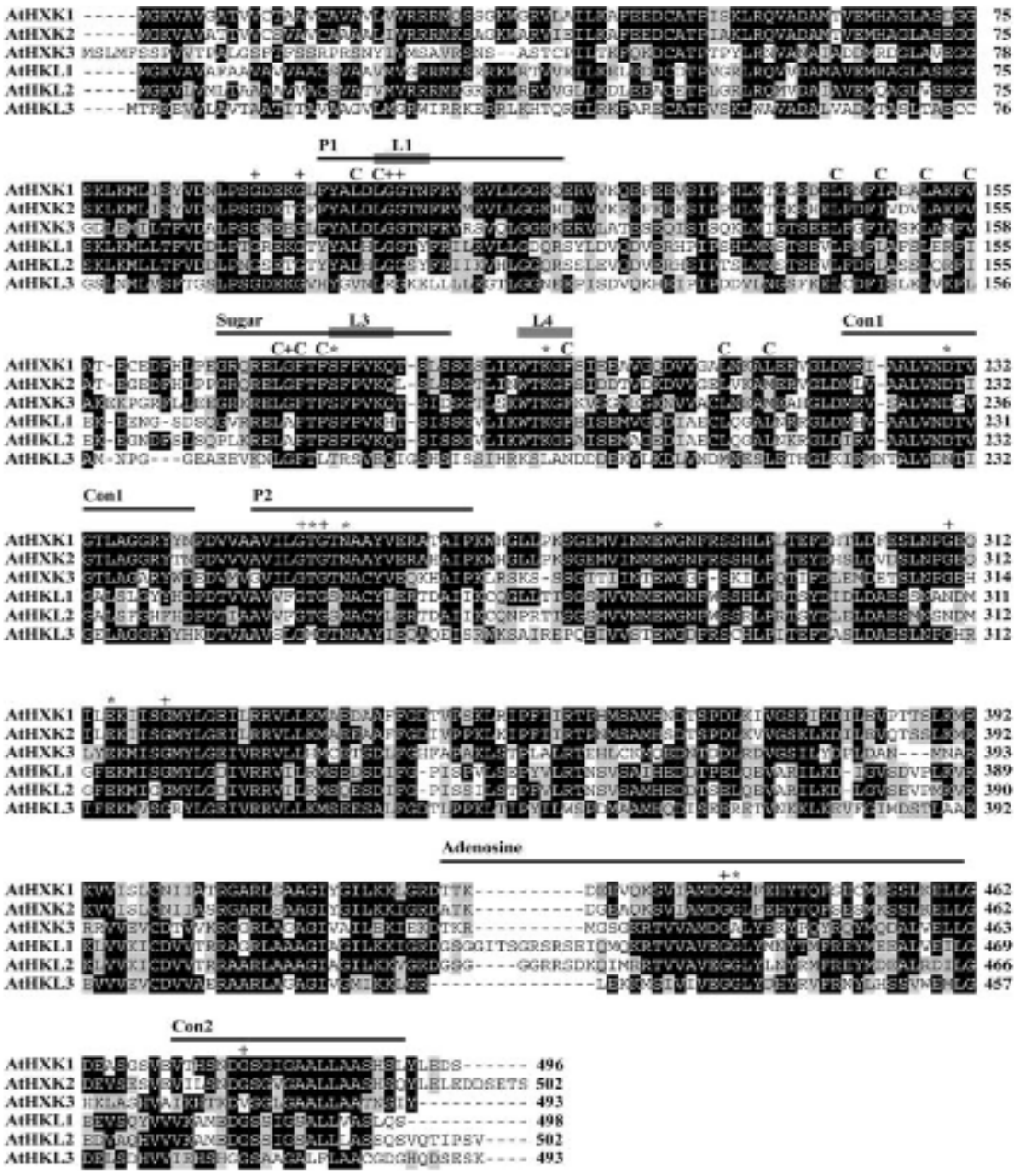

Figure 2.4 Amino acid sequence alignment for predicted Arabidopsis HXK family proteins. Sequences were aligned using ClustalW in BioEdit (Hall 1999). Annotations are based on regions homologous to yeast hexokinase II (Kuser et al. 2000). P1 = phosphate 1; Con 1 = connect 1; P2 = phosphate 2; Con 2 = connect 2; C = predicted hydrophobic channel amino acid; + = predicted conserved glycine residue; * = other predicted conserved residues (following AtHXK1 sequence, S177, K195, D230, T253, N256, E284, E315, and G441).

of 10 and 6 amino acids, respectively, at the adenosine binding site. These both also have relatively divergent phosphate 1 and connect 1 motifs. Within the core sugar binding motif (LGFTFSFP--Q--L/I), the sequence is best conserved among HXK1, HXK2, and HXK3, with a limited divergence in HKL1 and HKL2, but extensive divergence in HKL3 (see also Supplemental Table 1). However, with reference to the HXK1 sequence, the key glucose contact residues S177, N256, E284, and E315 are conserved in all of the HXKs, with the single exception of T175 for S177 in HKL3. Among the 4 key noted loops, loop 2 is not conserved at the level of amino acid identity between ScHXK2 and any of the AtHXKs, but the other 3 loops have varying levels of identity. Loops 1, 3, and 4 diverge substantially in HKL3, but are identical or very similar in all other AtHXKs, with respect to ScHXK2 (see also Appendix A). Among the 12 hydrophobic residues previously assigned to a channel in the small domain, these are either conserved or identical in all of the AtHXKs, with the exception of 2 different ones in HKL3 at F197 and L211 (Fig. 2.4). Also, among 8 key glycine residues thought to be located at the end of α-helices or β-sheets, these are all conserved identically among HXK1, HXK2, and HXK3 proteins. The HKL predicted proteins though do show some divergence in these features. This includes substitutions for G103 in HKL3, for G173 and G310 in HKL1 and HKL2, and for G479 in all 3 HKL proteins. Finally, there are 2 recognized catalytic residues in HXK1, K195 and D230. Both are conserved in all of the AtHXKs except for HKL3 (L194 and N230). In summary, the amino acid sequence analysis shows a broad pattern of conserved key motifs and residues among HXK1, HXK2, and HXK3 predicted proteins. HKL3 protein lacks many recognized residues important for sugar and

adenylate binding and for enzyme catalysis. In contrast, HKL1 and HKL2 proteins have most of the residues known to be important for sugar binding, but they do have a few noted key residue changes and also have an indel at the adenosine binding domain. Additionally though, HKL1 and HKL2 proteins do have changes in many residues relative to HXK1 protein, other than those specifically noted above.

Catalytic activities of native and modified AtHXK family proteins

We assessed the catalytic competency of HXK family proteins after transient expression of corresponding cDNAs in maize leaf protoplasts. In these experiments, HXK family genes were cloned as C-terminal fusions to double HA tags. Each HA tag is 10 amino acids and the double tag does not appear to interfere with protein catalytic activity as measured with different C-terminal tags (double HA, Flag, GFP; unpublished data). Protein expression first was monitored by direct labeling using ^{35}S-Met (Fig. 2.5 A) to adjust the amounts of transfected cDNAs to yield comparable amounts of expressed proteins for activity assays. In one approach to assess possible protein catalytic activities, we measured enzyme activities from protoplasts following their transient transformation with selected amounts of target cDNAs and an incubation period to allow the proteins to accumulate (Fig. 2.5 B). This approach was rapid, but does include as background the endogenous HXK activity that is present in the protoplasts. Nonetheless, we found that three of the six HXK genes encode proteins with glucokinase activity. HXK1 and HXK2 were both shown previously to have catalytic activity (Jang et al. 1997), while the finding

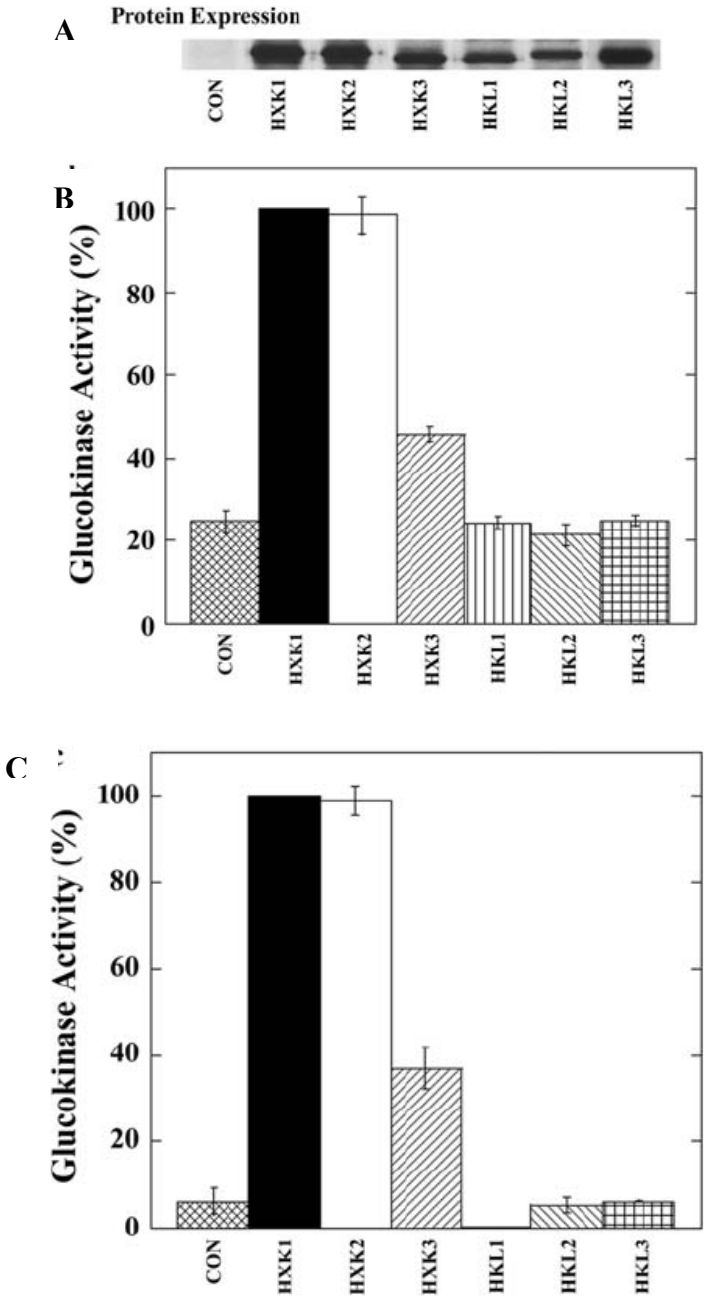

Figure 2.5 Glucokinase activity measured after transient expression of different cDNAs in protoplasts from greening maize leaves. (A) The fluorogram shows protein expression levels from all of the HXK cDNAs, following their labeling with ^{35}S-Met. In this gel image, band intensities ranged from 60% (HKL2) to 100% (HKL3), as measured using ImageJ software (http://rsb.info.nih.gov/ij/). Target cDNA amounts were slightly adjusted for better balanced expression in subsequent transfections. (B) Full length HXK-HA family proteins were expressed and assayed directly after protoplast lysis. (C) Full length HXK-

HA family proteins were expressed, then immunoprecipitated after protoplast lysis, and collected onto Protein A agarose beads prior to assay. Activity values are means ± SD of measured activities from 3-4 separate protoplast transfections with each construct. Activities are expressed as percent of HXK1. Notably, control values in **c** represent assay detection limits that are inherent when using enzyme-bound beads in an optical assay. CON = control, without transfected HXKs; K1 = HXK1; K2 = HXK2; K3 = HXK3; L1 = HKL1; L2 = HKL2; L3 = HKL3.

that HXK3 can phosphorylate glucose is novel. The three expressed proteins that apparently lack catalytic activity are designated, as mentioned earlier, as HKL proteins. Comparable results were obtained with 0.1 M fructose or increased glucose concentrations in the assay medium. That is, HXK3 can phosphorylate fructose also, while HKL1, HKL2, and HKL3 proteins cannot phosphorylate fructose either (data not shown).

To minimize the appreciable background HXK activity that is present in the protoplasts, we also assayed the expressed proteins after their immunoprecipitation from lysed protoplasts, captured using anti-HA antibody and Protein A agarose beads (Fig. 2.5 C). Using the washed beads allowed us to exclude most, if not all, of the endogenous activity and thereby better determine whether a given construct has any measurable catalytic activity. As shown, HXK1, HXK2 and HXK3 again all have easily measured glucose phosphorylation activity, while the three HKL proteins lack any such activity. As before, substitution of 0.1 M fructose for 2 mM glucose in the assay gave the same qualitative results (data not shown). While these protein expression assays are very useful for establishing whether a protein has catalytic activity, these preparations are not well suited for rigorous kinetic analyses.

The lack of glucose phosphorylation activity in the HKL proteins is not surprising in view of the key amino acid changes that were noted above, especially for HKL3. However, we asked whether the absence of catalytic activity in the HKL1 and HKL2 proteins might require a suite of changes relative to the active enzymes or whether there might be one or just a few key changes that are sufficient to make the enzyme inactive. A further examination of the amino acid sequences revealed that the HKL1 and HKL2 proteins have equivalent changes at 39 positions relative to conserved residues in the 3 proteins with catalytic activity. For example, in the core sugar binding motif, Gly173 is substituted with Ala in both HKL1 and HKL2. We therefore carried out site directed mutagenesis of both HXK1 and HKL1 in order to test whether key amino acid changes might compromise catalytic activity of HXK1 or might restore activity of HKL1. The target amino acids included Asn106 (located next to Loop 1), Gly173 (located within the sugar binding domain), Leu 251 (located within phosphate 2), insertion into HXK1 (at the corresponding position, K427) of the additional 10 amino acids present at the adenosine domain of HKL1, and removal of the 10 amino acid adenosine indel from HKL1. As a possible negative control, Cys159 of HXK1 was changed to Glu. Cys159 is one of only three amino acids among all six HXKs in which there is variation among either five or all six of the amino acids. We also included the previously altered construct S177A, which has no catalytic activity (Moore et al. 2003). In this experiment, expressed proteins were again collected onto agarose beads in order to minimize the background glucokinase activity endogenous to the protoplast. Most of the amino acid changes did substantially impact the glucokinase activity (Fig. 2.6).

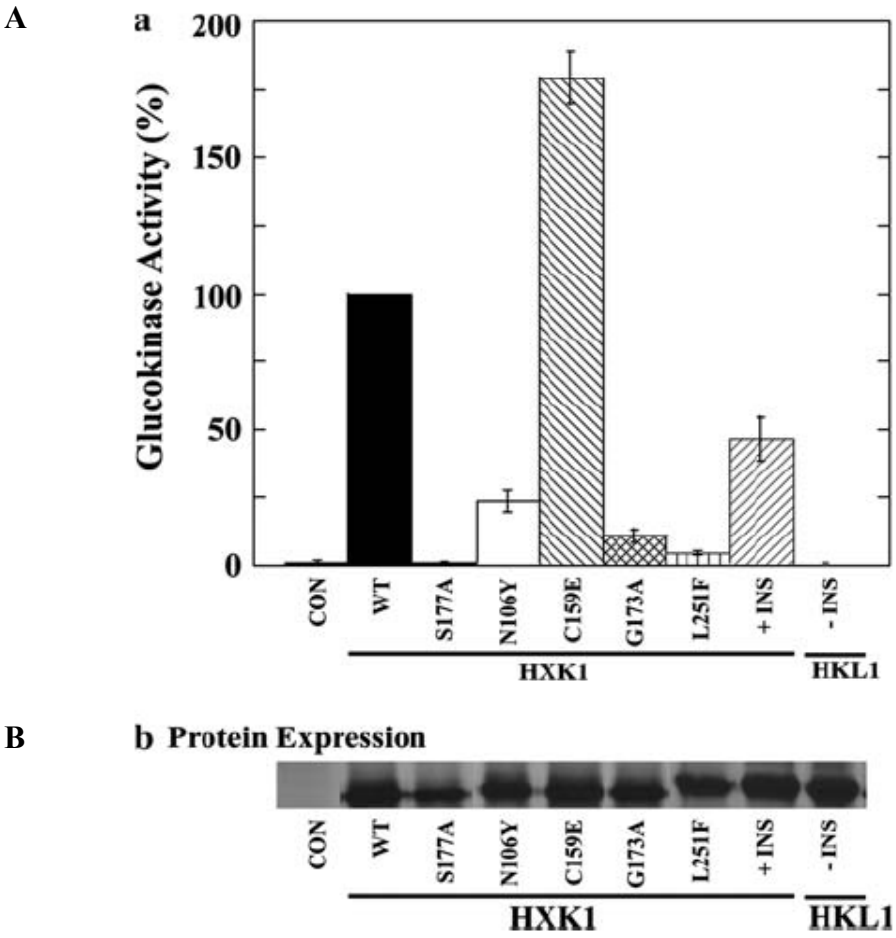

Figure 2.6 Glucokinase activity of HXK1 and HKL1 proteins modified by site directed mutagenesis. (A) Modified proteins were expressed in protoplasts from greening maize leaves, then immunoprecipitated after protoplast lysis, and collected onto Protein A agarose beads prior to assay. Activity values are means ± SD of measured activities from 3-4 separate protoplast transfections with each construct, expressed as percent of WT HXK1. (B) The fluorogram shows protein expression levels following labeling with ^{35}S-Met. Protein bands correspond precisely as labeled in (A). In this gel image, band intensities ranged from 75% (S177A) to 100% (C159E), as measured using ImageJ software. HXK1 + INS = HXK1 with a 10 amino acid insertion at the adenosine domain; HKL1 – INS = HKL1 with deleted 10 amino acid indel at the adenosine domain; see text for further details.

Changing N106Y, G173A, and L251F reduced enzyme activity by 75, 90, and 95% respectively. The 10 amino acid insertion into HXK1 reduced activity about 55%, and

removal of the indel from HKL1 did not restore any detectable activity. Surprisingly, the C159E mutation stimulated activity 2-fold. We observed the same results with all constructs expressed in parallel transfections in which enzyme activities were measured directly from the lysed protoplasts (data not shown). From these data, we suggest that there likely are many non-conserved amino acids in the HKL proteins that could each compromise catalytic activity. However, we cannot exclude the possibility that particular combinatorial changes among all of those present might have compensatory effects that otherwise would result with changes to single key amino acids.

Comparison of HXK family sequences from A. thaliana and A. lyrata

The *A. lyrata* genome is currently being sequenced through the Joint Genome Institute of the Department of Energy (http://www.jgi.doe.gov/index.html). The North American species, *A. lyrata*, is thought to have diverged from *A. thaliana* about 5 million years ago (Koch and Matschinger 2007). In contrast to *A. thaliana*, *A. lyrata* is self-incompatible and out-crossing. We have compared the HXK family member gene sequences between *A. thaliana* and *A. lyrata* in order to identify possible regions of gene orthologs that might be undergoing differential selection. For all of the AlHXK family members, the available sequence data were sufficient to enable us to identify the homologous exons, introns, and splice sites for each gene except *AlHXK3*. In the case of *AlHXK3* direct sequencing resolved a discrepancy in the first draft sequence within exon 6. The HXK gene structures are very similar in *A. lyrata* as

Table 2.2 Comparisons of HXK amino acid sequences from *A. thaliana* and *A. lyrata*

	% Identity	% Similarity	# of Gaps
HXK1	98	99	0
HXK2	97	98	0
HXK3	89	94	4
HKL1	97	99	0
HKL2	98	99	0
HKL3	93	95	10

compared with those in *A. thaliana* (Fig. 2.1, Appendix B). For example, *AlHKL3* has many short introns, as does *AtHKL3*. The most notable difference in overall gene structure is that intron 1 of *AlHKL1* is about 200 nucleotides shorter than intron 1 of *AtHKL1*.

The shared predicted amino acid identities were generally very high between all of the orthologues (e.g. > 97%) except for HXK3 and HKL3 (Table 2.2, Appendix C). For example, the predicted HKL1 and HKL2 proteins from *A. lyrata* have exactly the same indels at the adenosine binding domain as the othologous proteins from *A. thaliana*. In contrast, the HXK3 and HKL3 gene pairs have lower percent identity values of 89 and 93, respectively, and have some gaps in their aligned sequences. A phylogenetic tree based on all 12 genes indicates that the orthologous genes are much more related to each other, than to other family members (Appendix B). These relationships also support a previous more global phylogenetic analysis that includes additional plant HXK sequences

(Cho et al. 2006a). Notably, the HXK1 and HXK2 protein homologs have accumulated fewer amino acid changes from the nearest common ancestral sequence than have the HKL1 and HKL2 protein homologs.

One approach to test for possible differential rates of sequence evolution within a gene pair is to compare rates of synonymous and nonsynonymous nucleotide substitutions. Because of multiple indels particularly in the 3' end of the *HKL1* and *HKL2* genes, analyses of genomic DNA sequences were only possible for comparing specific *A. thaliana* and *A. lyrata* orthologs (for example, *AtHKL1* versus *AlHKL1*). Although it would be informative to consider divergence of the sequences coding for exons plus introns between the two species, the presence of non-triplet indels made this not feasible using the polymorphism and divergence analyses of DnaSP since any non-triplet indels lead to false reading frames in the sequence containing the possible sequence deletion. For example, the *HKL1* and *HKL2* genes have 13 indels total, of which only 1 is a triplet, though all of the indels do occur in groups that add up to a triplet. After analyzing the coding sequences for possible codon usage bias (none detected, data not shown), exon sequences for gene orthologs were then examined.

To test for the potential contribution of selection on sequence divergence between species and genes, the rates and patterns of sequence divergence of each member of the HXK family of genes were tested between *A. thaliana* and *A. lyarata* (Fig. 2.7). K_A/K_S ratios of less than 1 indicate coding regions that are selectively constrained (purifying selection with higher rates of synonymous versus nonsynonymous mutation). Regions

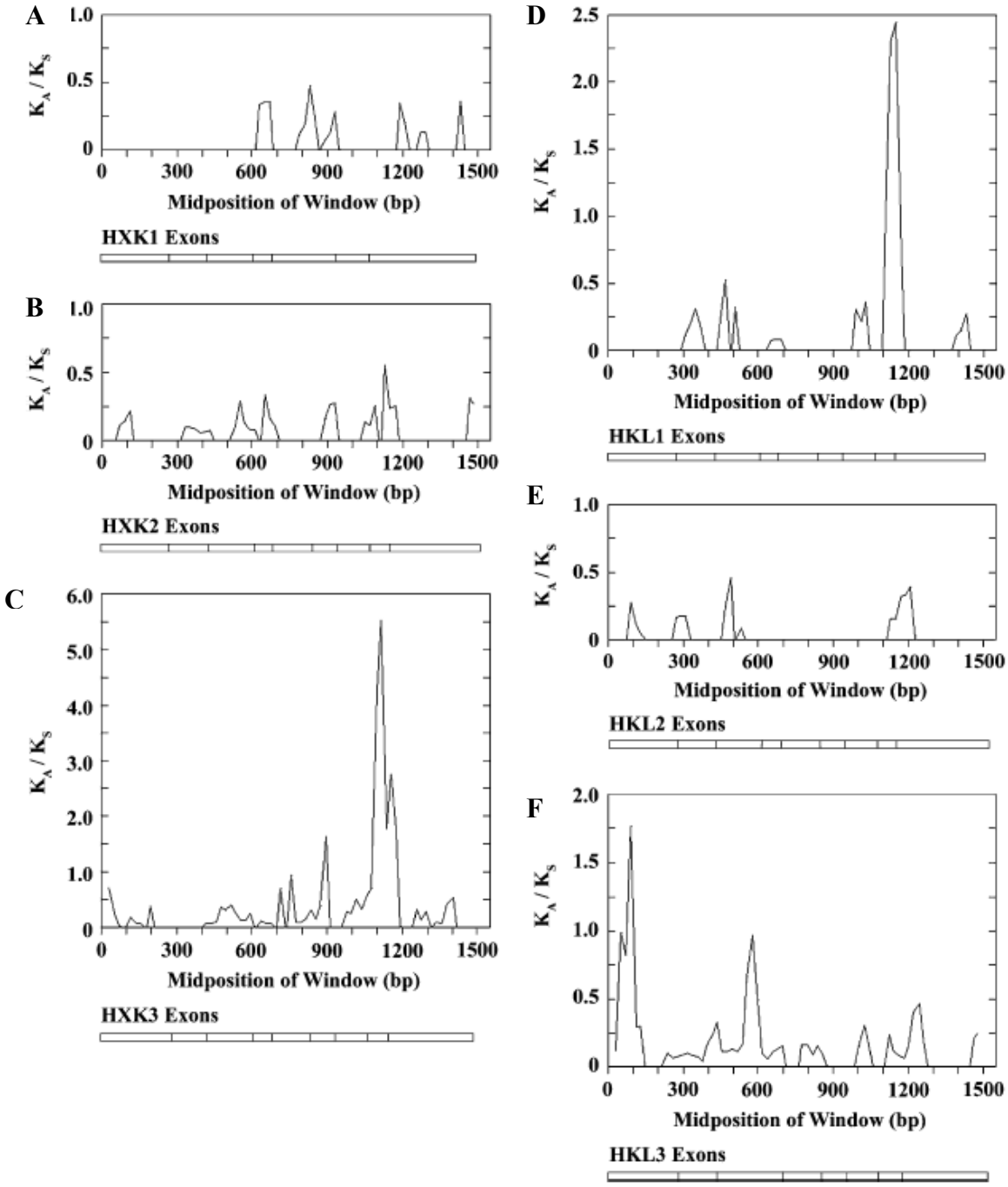

Figure 2.7 Sliding window analyses of K_A/K_S of selected HXK and HKL loci. Analyses were done using DnaSP (Rozas et al. 2003) as described further in "Materials and methods". (A) HXK1. (B) HXK2. (C) HXK3. (D) HKL1. (E) HKL2. (F). HKL3. Inserted exon bars correspond in length to predicted numbers of amino acids. bp = base pairs. Undefined estimates of K_A/K_S (because $K_S = 0$ at these sites) occurred in

the following sliding windows: HXK1 (at midpoints 710, 730, 750, 1170, 1310, 1330, 1350, 1370, 1390, and 1410 bp), HXK2 (at midpoint 630 bp), HXK3 (at midpoints 216, 236, 256,276, 336, 356, 376, 396, 736, 916, 936, 1196, and 1422 bp), HKL1 (at midpoints 490, 1070, and 1090 bp), HKL2 (at midpoints 510 and 1230 bp), and HKL3 (at midpoint 757 bp)

with values similar to 1 indicate neutral evolution. Regions having ratios greater than 1 indicate a higher rate of nucleotide substitutions that change the amino acid sequence versus synonymous mutations (adaptive selection; for further descriptions see Parmley and Hurst 2007). All HXK family genes have consistently low K_A and K_S values and most have K_A/K_S values much lower than 1: HKL1 and HKL2 have overall ratios of 0.08 and 0.05, respectively; HXK1 and HXK2 have ratios of 0.09 and 0.06, respectively; HKL3 has a ratio of 0.12; and, HXK3 has a higher ratio of 0.44. These overall low values indicate that all members of the HXK gene family are active in these two sister Arabidopsis species and are being selectively constrained to their current amino acid sequences. To further extend this analysis, sliding windows of K_A/K_S were calculated using window sizes of 60 nucleotides and a step size of 20 nucleotides (Fig. 2.7). The window size roughly correlates with the size of some structural elements of the proteins. Most regions of the presented genes are constrained to 0 across much of the gene and only short regions have increased K_A/K_S ratios. The primary exception is a large peak in K_A/K_S value that corresponds to exon 8 in both *HKL1* and *HXK3*. This region is somewhat upstream of the noted large indel of HKL1, but in exon 8 there are no apparent functional protein motifs or key amino acids that have been described. K_A/K_S ratios of the *HXK2* gene pair were similar in profile to *HXK1*, while analysis of *HKL3* showed a prominent peak in K_A/K_S in exon 1.

Discussion

In this study, we have reported expression characteristics of the six HXK family members of *A. thaliana*. Among the HXK proteins that have hexose phosphorylation activity, AtHXK3 has not been previously recognized. We have shown that AtHXK3 is a stromal-localized protein (Figs. 2.3), likely expressed at low abundance primarily in roots, stems, and siliques (Fig. 2.2). As noted with other plastid HXKs (Cho et al. 2006a; Kandel-Kfir et al. 2006), the presence of the transit peptide in AtHXK3 might have led to this protein not being identified in a previous complementation study of yeast cells deficient in glucose phosphorylation activity, using Arabidopsis cDNAs (Jang et al. 1997). The organ distribution of AtHXK3 is similar to that observed for tobacco plastid NtHXK2 from promoter-GUS studies (Giese et al. 2005). NtHXK2 expression was further shown to be localized in specialized tissues such as guard cells, root tips, xylem parenchyma and the vascular starch sheath. Accordingly, NtHXK2 was suggested to function primarily in starch degradation (Giese et al. 2005). On the other hand, LeHXK4 expression in plastids is perhaps relatively more wide-spread among different tomato tissues and its expression in fruits is not associated with starch degradation (Kandel-Kfir et al. 2006). As noted in both of these studies and elsewhere (Olsson et al. 2003), plastid HXKs can have an important function also in metabolizing imported glucose for production of erythrose 4-phosphate to supply the shikimic acid pathway for synthesis of some 2^{o} metabolites. Since AtHXK3 mRNA is expressed mostly in sink tissues (Fig.

2.2), we suggest that this protein might have a more pronounced role in phosphorylating glucose that is imported into the plastid for biosynthetic processes.

Three of the six HXK family genes in *A. thaliana* encode proteins that do not phosphorylate glucose or fructose (Figs. 2.2, 2.5). Therefore we suggest that non-catalytic HXKs most likely do exist in plants and that particular HKL proteins might occur in most tissues. Their lack of catalytic activity would account for these also not being identified in the previous yeast complementation study by Jang et al. (1997). All three encoded HKL proteins are about 50% identical to HXK1 and they do cross-react well with a polyclonal anti-HXK1 antibody (Karve and Moore, unpublished data). The observed presence of HXK1-related antigen in leaves of *gin2-1* (Fig. 2.4) supports our suggestion that the HKL proteins might be expressed in leaves and elsewhere in Arabidopsis.

Several of the reported expression characteristics of HKL1 and HKL2 proteins indicate they might have a regulatory function in *A. thaliana*. First, transcripts for both genes were present at similar levels in all organs examined (Fig. 2.2). Function in a broad tissue context could be important if these proteins somehow affect HXK-dependent glucose signaling or possibly other wide-spread regulatory processes. Second, both HKL proteins are targeted to mitochondria, as predominantly is the case for AtHXK1 (Fig. 2.3). We have previously shown that mitochondrial targeted HXK1 can bind to porin in the outer membrane and can mediate at least some aspects of glucose signaling (Balasubramanian et al. 2007). In this regard, it will be important to establish whether these proteins might interact with AtHXK1. A third important finding is that AtHXK1

catalytic activity is readily compromised by any of a number of single amino acid changes (Fig. 2.6, Moore et al. 2003). Since there are possibly many such changes in the 1^{o} amino acid sequences of the HKL proteins (Fig. 2.4, Appendix A), we surmise that the presence of catalytically defective HXKs is not simply the result of a chance mutation. Yet to be established is whether these proteins can bind glucose and with what affinity. On the one hand, the G173A change in the sugar binding domain of AtHKL1 and AtHKL2 could impact their ability to bind glucose, since the corresponding change in AtHXK1 did substantially reduce catalytic activity (Fig. 2.6). However, in the absence of a detailed structure analysis or ligand binding assays, one cannot rule out that glucose or even other sugars might bind with sufficient affinity in the HKL proteins as to be biologically relevant. From their primary sequence analysis, we predict that both HKL1 and HKL2 proteins do have extensive conformational flexibility as inferred by the presence of conserved key loop motifs and most of the important glycine residues at the ends of structural elements (Appendix A). That is, the primary recognized elements required for a glucose-dependent conformational change in protein structure are largely conserved and could be exploited by cell regulatory mechanisms. The described expression and sequence characteristics support the hypothesis that the HKL1 and HKL2 proteins might affect glucose signaling or related processes.

The AtHKL3 protein has quite different expression and sequence characteristics from the other two non-catalytic AtHXKs. Among plant organs that we examined, HKL3 mRNA expression was restricted only to flowers (Fig. 2.2). This finding largely supports the conclusion by Claeyssen and Rivoal (2007) from a survey of transcriptional

profiling experiments, that *AtHKL3* is expressed most abundantly in male reproductive parts of the flower. Interestingly, among rice HXKs, OsHXK10 is expressed in pollen, but only there (Cho et al. 2006a). Our analysis of the predicted amino acid sequence of AtHKL3 (Fig. 2.4, Appendix A) indicated that it would not likely bind glucose or ATP as substrates, in contrast to other HXK family proteins, and that it does not have the conserved mobile loops or elements required for structural flexibility that are characteristic for this family of proteins. We suggest that this protein might have been recruited by evolutionary processes to have a much different function than those of other family members. Notably, cluster analysis of amino acid sequences of rice and Arabidopsis HXKs, indicate that the AtHKL3 protein occurs as an isolated group established prior to the separation of monocots and dicots (Cho et al. 2006a).

Non-catalytic HXKs have been identified in a variety of fungi including *Saccharomyces cerevisiae* and *A. nidulans* (Katz et al. 2000; Bernardo et al. 2007), in *Drosophila melanogaster* (Kulkarni et al. 2002), and now in *A. thaliana*. Previous phylogenetic analysis suggests that the non-catalytic HXKs evolved independently in different lineages (Bernardo et al. 2007). Nonetheless, there are some intriguing comparisons in the primary sequences of these proteins. The fungal and fly HKL proteins were noted as having a number of altered residues in the sugar binding domain, as well as many of the fungal proteins having an indel of about 20-25 amino acids at the adenosine domain (Bernardo et al. 2007). Arabidopsis HKL1 and HKL2 proteins have much better conserved sugar binding domains (one or two substitutions) and have a similarly positioned indel of 6-10 amino acids in the adenosine domain. The predicted

adenosine domain in the Arabidopsis catalytic HXKs extends eight amino acids towards the N-terminus relative to the adenosine domain in yeast HXK2 (Kuser et al. 2000). The prominent indels in the AtHKL1 and AtHKL2 proteins occur within this extension and are located four amino acids to the N-terminal side of the corresponding indel in *A. nidulans* HXKC and HXKD proteins. Whether these indel sequences in AtHKL1 and AtHKL2 actually function in adenosine binding is uncertain. Alternatively, these indels might be important for possible regulatory functions of the non-catalytic HXKs. Among the rice HXK family, all 9 expressed members are thought to have catalytic activity, based on their ability to complement the HXK-deficient, triple mutant of yeast (Cho et al. 2006a). However, OsHXK3 and OsHXK10 do form a distinct phylogenetic group with AtHKL1 and AtHKL2 proteins (Cho et al. 2006a). Upon sequence inspection, both rice proteins also contain a similar indel of nine amino acids at the same aligned position near the beginning residue of the predicted adenosine domain (data not shown). The sugar binding domains of OsHXK3 and OsHXK10 do not have the homologous substitution of A for G as do AtHKL1 and AtHKL2 (LAFTFSFP--Q), though these domains do have 1 or 2 changes elsewhere.

The different expressed features within the HXK family of a given plant or fungal species reflect some remarkable apparent evolutionary trends. In both *A. thaliana* and *S. cerevisiae*, there occurs at least one moonlighting HXK which has separable functions as both a metabolic catalyst and a glucose sensor/transducer (Moreno and Herrero 2002; Moore et al. 2003). Additionally, there occur proteins within the families that have apparent specialized metabolic roles or specialized regulatory roles. The metabolic only

catalysts include yeast HXK1 and glucokinase1 (Santangelo 2006), and we suggest might also include plant plastid HXKs. Among non-catalytic HKL proteins, there are two HKL proteins in *S. cerevisiae* (Bernardo et al. 2007), one of which, EMI2, is required for induction of a meiosis-specific transcription factor (Daniel 2005). In *A. nidulans*, the non-catalytic proteins AnHXKC and AnHXKD both are thought not to have a role in glucose signaling, but genetic evidence indicates that instead both are negative regulators for a secreted extracellular protease during carbon starvation (Bernardo et al. 2007). AnHXKC is associated with mitochondria, while AnHXKD is present in the nucleus. As noted above, it is yet to be demonstrated whether the Arabidopsis HKL proteins do have specialized regulatory functions. Notably, AnHXKD is transcriptionally induced by carbon starvation, yet neither AtHKL1 nor AtHKL2 are apparently induced by starvation conditions (see Supplementary Tables in Baena-González et al. 2007).

While non-catalytic HXKs are present in the relatively distant lineages of fungi and at least Arabidopsis, we also interrogated the HXK nucleotide sequences of *A. thaliana* and *A. lyrata* to test for possible different relative rates of evolution among the respective orthologs. HXK3 has a higher K_A/K_S value than do all the other loci examined. This indicates those amino acids sequences are less constrained overall for accumulating changes. Notably, the divergence in amino acid sequences between AtHXK3 and AlHXK3 orthologs also is greater than for the other catalytic HXKs (Table 2.2). We suggest that the *HXK3* gene is evolving at an increased rate relative to the other genes for the catalytic HXK proteins and for the HKL proteins. In contrast, among the orthologs for HXK1, HXK2, HKL1, and HKL2, the genes are evolving overall at similar

rates. However, both HKL1 and HXK3 orthologs have a pronounced peak of K_A/K_S ratio within exon 8 (Fig. 2.7). This indicates a possible adaptive selection process for these sequences. The significance of this observation is not yet clear, but does warrant investigation.

For the global plant HXK gene family, it will be interesting to establish whether the noted indel that occurs in Arabidopsis HKL1 and HKL2 might be useful as a molecular marker for phylogeny studies. To that end, it is important to verify if OsHXK3 and OsHXK10 actually do have catalytic activity as suggested (Cho et al. 2006a), since their sequences also contain a similar indel at the homologous position as occurs in the reported non-catalytic HKL proteins.

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CHAPTER THREE

FUNCTION OF ARABIDOPSIS HEXOKINASE-LIKE 1 AS AN EFFECTOR OF PLANT GROWTH

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Abstract

A recent analysis of the hexokinase (HXK) gene family from Arabidopsis has revealed that three of the six genes encode proteins that lack catalytic activity. In this study a reverse genetics approach was used to understand the function of hexokinase-like 1 (HKL1), one of the three non catalytic HXKs from Arabidopsis. HKL1 overexpression in Ler (HKL1-HA) resulted in severe reduction in rosette size under long photoperiod, decreased growth of seedling hypocotyls, a reduced seedling sensitivity to growth on plates with 6% glucose, and a reduced sensitivity to auxin induced lateral root formation. Conversely a HKL1 knockout, *hkl1-1*, had reduced rosette size under short photoperiod, longer seedling hypocotyls, and increased seedling sensitivity to growth on plates with 6% glucose. The phenotypes of HKL1-HA were mostly similar to those reported for a hexokinase null mutant, *gin2-1*. HKL1 overexpression in *gin2-1*, however, did not generally affect the phenotypic responses of the parental line. Therefore, we suggest that HKL1 is a negative regulator of plant growth, but that its effect largely depends on presence of HXK1.

Since by protoplast assays, HKL1 did not affect the activity of glucose regulated promoters we conclude that HKL1 likely is not directly involved in glc signaling. The expression and regulation of HKL1 was also investigated by analysis of *pHKL1-GUS* transgenic plants. HKL1 promoter was strongly expressed in vascular tissue and was responsive to ABA, ethylene and cytokinin. Combining the results of phenotypic assays and promoter regulation, we suggest that HKL1 somehow mediates cross talk between glc and other plant hormone response pathways.

Introduction

To respond to environmental and metabolic cues, complex regulatory networks have evolved in all living organisms which enable them to sense nutrient status and regulate their gene expression. Glucose (glc) is one example of an important metabolic intermediate that functions also as a signaling molecule to regulate gene expression in a variety of organisms (Towle 2005; Rolland et al. 2006; Gancedo 2008). In the case of plants, glc affects the expression of more than 1000 genes (Price et al. 2004; Osuna et al. 2007), many of which influence phytohormone response pathways that control plant growth (*e.g.*, Gibson 2004). In fact, many mutants of plant glc signaling are alleles of genes with defined roles in ABA or ethylene biosynthesis and/or their signaling networks (Leon and Sheen 2003; Rognoni et al. 2007).

Genetic and biochemical evidence supports independent roles in plant glc sensing and phytohormone responses for both hexokinase (HXK) and the plant regulator of G-protein signaling (RGS; Rolland et al. 2006). Arabidopsis HXK1 functions as a glc sensor and modulates plant growth at many different developmental stages (Moore et al.2003). For example, AtHXK1 mediates cell cycle arrest in seedlings grown in the presence of high levels of exogenous glc (Jang et al. 1997) by a process not dependent on enzyme catalytic activity (Moore et al. 2003). Null mutants of AtHXK1 have reduced shoot and root growth, increased apical dominance, delayed flowering, and altered sensitivity to both auxin and cytokinin (Moore et al. 2003). Arabidopsis RGS1 is an atypical RGS protein having both a 7-transmembrane domain as well as a functional

GTPase activation domain (Chen et al. 2003; Johnston et al. 2007). AtRGS1 is suggested to function as a glc binding protein that can attenuate cell division in primary root apical meristems by activating the GTPase activity of GPA1 (Chen et al. 2006). However, even though seedlings of null mutants of AtRGS1 and AtHXK1 both show a similar glc-dependent developmental arrest, their hypocotyl elongation response to light or dark are opposite to each other (Chen et al. 2003; Moore et al. 2003). Similar to HXK1 though, plant heterotrimeric G-proteins also affect a diverse array of developmental and hormone responses (Perfus-Barbeoch et al. 2004).

Plant HXKs are encoded by a modest family of about 5-10 genes (Claeyssen and Rivoal, 2007). HXK proteins are reported to occur in the cytosol, mitochondria, plastids, nuclei and Golgi (Balasubramanian et al. 2007; and, references therein). AtHXK1 is predominantly associated with the mitochondria, but also reportedly can occur in the nucleus (Cho et al. 2006b). There is evidence that from both locations, AtHXK1 can regulate gene and/or protein expression, but there are questions regarding both scenarios (see Balasubramanian et al. 2008). All three Arabidopsis HXK-like proteins (HKL) were shown to be associated with mitochondria (Heazelwood et al. 2004; Karve et al. 2008).

A recent analysis of the Arabidopsis HXK gene family revealed that three of the six members lack catalytic activity when assayed with varying concentrations of glc or fructose (Karve et al. 2008). Sequence analysis further showed that the three HKL proteins are about 50% identical to HXK1, with many distributed amino acid changes. In AtHKL3, sequence divergence among key domains and other functional residues is so extensive that the protein is thought not to bind either glc or ATP. However, recognized

functional motifs are much better conserved in AtHKL1 and AtHKL2, and their glc binding domains are predicted to be functional (Karve et al. 2008). The basis for the lack of catalytic activity in these proteins was attributed to any of a number of changes throughout the primary sequences, and not with a specific single amino acid change (Karve et al. 2008).

Non-catalytic HXKs have been reported in fungi and likely occur commonly among higher plants (Virnig and Moore, unpublished data). The fungal HKL proteins have divergent roles including one as a meiosis-specific transcription factor in *Saccharomyces cerevisiae* (Daniel 2005) and others as regulators of the carbon starvation response in *Aspergillus nidulans* (Bernardo et al. 2007). Despite the reports of the presence of HKL proteins in evolutionarily diverse species, their lack of catalytic activity and possibly low level of expression, at least in plants (Karve et al. 2008), has led to a general lack of understanding of their functions. In this study, we have used a reverse genetics approach to ask whether AtHKL1 might have a role in plant growth, perhaps in a fashion related to that of the primary glc sensor protein, AtHXK1. We have therefore examined phenotypes from gain-of-function Arabidopsis plants, from an identified mutant line with a T-DNA insertion in HKL1, and from HKL1 promoter-GUS transgenic lines. We show that AtHKL1 does have a substantial role in plant growth that is largely dependent on the presence of AtHXK1. Furthermore, the HKL1 promoter is strongly expressed in vascular tissues and is responsive to multiple plant hormones. The observed growth and glc response phenotypes indicate that AtHKL1 has a crucial role in plant growth and development, but an indirect role in glc signaling.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0), ecotype Landsberg *erecta* (Ler), and a Col line with a T-DNA insertion within the *HKL1* locus (WISCDSLOX383A5, *hkl1-1*) were obtained from the Arabidopsis Biological Resource Center (Ohio State University); *gin2-1*, HXK1-HA, HXK1-FLAG, and *tir1* were as previously described (Moore et al. 2003). Lines homozygous for the T-DNA insertion in the *HKL1* gene (AGI code, At1g50460) were selected by PCR genotyping using the following primers: p745 (5'AAC GTC CGC AAT GTG TTA TTA AGT TG3') and HKL1 A5RP (5'CCG TGT TAT CTG AGC CTT ACG 3') for the T-DNA insertion allele; and, HKL1 A5LP (5' TGC AAA CAA ATT TAA CGG CTC 3') and HKL1 A5RP for the WT allele. The insertion position in the *hkl1-1* mutant was mapped by sequencing the PCR product obtained by the primers L1 WLP (5'TGC AAA CAA ATT TAA CGG CTC 3') and L1 WRP (5' CCG TGT TAT CTG AGC CTT ACG 3'), using *hkl1-1*genomic DNA as template. Seeds of maize (*Zea mays* L.) were purchased (Seed Genetics).

Arabidopsis seeds were surface sterilized and stratified for 2 days at 4 ^{0}C as in Jang et al. (1997) and grown either in soil in a growth chamber (12 h photoperiod, 125 μmol $m^{-2}s^{-1}$ at 22 ^{o}C/20 ^{o}C day/night temperature) or on 1X MS agar plates (modified basal medium with Gamborg vitamins; PhytoTechnology Laboratories) containing 0.7%

phytagar (Caisson Laboratories), pH 5.7, under constant light (30 μmol $m^{-2}s^{-1}$). Hypocotyl elongation assays were done as described earlier (Moore et al. 2003). In one experiment, plants were chamber grown under either short day (SD, 8 h) or long day (LD, 16 h) photoperiods.

For assay of auxin induced lateral root formation, Arabidopsis seeds were grown on 1X MS plates supplemented with 5 μM 1-naphthylphthalamic acid (NPA) for 5 d and then were transferred to MS plates ± 0.1 μM naphthalene acetic acid (NAA), as in Chen et al. 2003. For glc repression assays, seedlings were grown on 1X MS plates supplemented with 3, 4, 5, 6, or 7% glc or 6% mannitol for 7 d under constant light.

Plasmid and transgenic constructs

RBCS-LUC, *ASN1-LUC*, and *UBQ10-GUS* constructs were described previously (Schaffner and Sheen, 1991; Balasubramanian et al. 2007). The previous clone of *HKL1* with a double hemoagglutinin (HA) tag (Karve et al. 2008) was subcloned with a C terminal FLAG tag into a HBT vector (Moore et al. 2003), then transferred into pCB302 binary vector (*bar* selection marker; Xiang et al. 1999) using Bam HI and PstI cloning sites. For cloning the HKL1 promoter, a 3098 bp fragment upstream of the start codon was PCR amplified using the following primers: L1pGUSFP (5' CCC AAG CCT GGG CAG CGA GCT GTC AAA CTG GGG A 3') and L1PGUSRP (5' GCT CTA GAT GCC CCA AAA CAG AAC CAA AAA GAC A 3'). The promoter was cloned into the binary vector pSMAB704 (*bar* selection marker; Igasaki et al. 2002), using HindIII and SmaI

cloning sites upstream of the β-glucoronidase (GUS) gene. The identities of all clones were verified by DNA sequencing.

Binary constructs were introduced into *Agrobacterium tumifaciens* GV3101 by electroporation. Arabidopsis plants of Col-0, Ler or *gin2-1* were transformed with Agrobacterium using the floral dip method (Clough and Bent 1998). Transformants were selected for herbicide resistance (200 μM glufosinate ammonium, Rely 200, Bayer Crop Science). Seeds of transgenic lines segregating 3:1 for herbicide resistance in the T_2 generation were selected for isolating homozygous lines. Seeds from two or more T_3 lines homozygous for the single insert were used for subsequent experiments.

RT-PCR analysis, immunoblots, and gluokinase activity assays

Total RNA was isolated from whole seedlings using the RNeasy plant kit (Qiagen). One μg of total RNA was converted to cDNA using the Protoscript II RT-PCR kit (New England BioLabs) according to the manufacturer's instructions. PCR primer sequences for the *HXK1, HKL1* and *UBQ5* were described previously (Karve et al. 2008). The templates amounts were first titrated to balance the *UBQ5* expression and corresponding template amounts were used thereafter with varying PCR cycle numbers.

Total soluble proteins were extracted as described before (Karve et al. 2008). The protein concentration in the leaf extracts was measured by Coomassie blue (BioRad). Equal amounts of proteins were electrophorsed by SDS-PAGE and transferred on to Immobilon-P (Millipore) membrane. The membranes were probed with monoclonal anti-

HA (Roche) or anti-FLAG M_2 (Sigma-Aldrich) or polyclonal anti-HXK1 (Moore et al. 2003) antibodies, followed by incubation with HRP conjugated secondary antibody, and detection by chemiluminescence (SuperSignal West Pico, Pierce Biotechnology). Glucokinase activity was measured directly from leaf extracts or from lysates of transfected maize protoplasts (Karve et al. 2008).

Protoplast transient expression assays

Leaves of greening maize seedlings or Arabidopsis plants (Col or *hkl1-1*) were used as a source of protoplasts for protein expression and signaling assays (Jang and Sheen, 1994; Hwang and Sheen, 2001). Protoplasts were transfected using polyethylene glycol (Yoo et al. 2007), with promoter constructs for *RBCS-LUC* (4 μg), *ASN1-LUC* (6 μg), or *UBQ10-GUS* (2 μg) as in Balasubramanian et al. (2007). Protoplasts were co-transfected as indicated with effectors *HKL1-HA* (8 μg) and/or *HXK1-HA* (6 μg). An empty vector was included to maintain a balanced concentration of DNA during transfections. Transfection efficiencies were routinely > 60% as determined using WRKY-GFP (Balasubramanian et al. 2007). Following transfection, protoplasts were incubated in the dark for 90 min, then treated with 2 mM glc and incubated in the light for 6-8 h at 30 μmol $m^{-2}s^{-1}$. Protoplasts were collected by low speed centrifugation. After resuspending in lysis buffer, GUS and LUC activities were measured as described previously (Balasubramanian et al. 2007). Promoter activities are expressed as relative LUC/GUS values normalized to non-glc treated control samples.

Histochemical GUS staining and fluorometric GUS assays

Histochemical staining of transgenic Arabidopsis plants containing pHKL1-GUS fusion constructs was performed as described by Crone et al. (2001). The plant tissue was incubated in GUS staining buffer containing 25 mg ml^{-1} of X-Glc (Gold BioTechnology) for 2-4 h and destained with 95% ethanol for 6-8 h. For measuring total extractable GUS activity, tissues were ground in extraction buffer containing 50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β-mercaptoethanol. The fluorogenic reaction was carried out in 100 µl of extraction buffer plus 1 mM 4-methyl umbelliferyl glucuronide (MUG, Sigma-Aldrich) at 37 ^oC for indicated times, before stopping with 300 µl of 0.2 M Na_2CO_3. Fluorescence was measured in a 96-well microtiter plate format using a GENios spectrophotometer (Phenix Research Products) at 360 nm excitation wavelength and 465 nm emission wavelength. A standard curve was made using 0.1-1 µM 4-methyl-umbelliferone (Sigma-Aldrich) and used to determine sample GUS activity.

In one experiment, transgenic seeds of pHKL1-GUS expression were grown on 1X MS plates for 7 d, then transferred to liquid MS medium for 4 h with 10 µM indoleacetic acid (IAA), 1 µM abscisic acid (ABA), 50 µM 1-aminocylcopropane-1-carboxylic acid (ACC), or 10 µM zeatin (hormones from Sigma-Aldrich). Both treated and control seedlings were analyzed for GUS staining and extractable GUS activity as described above.

Light microscopy

Light microscopy was used to view and capture images for some routine seedling pictures as well as for the GUS stained seedlings or tissues, using a Nikon SMZ1500 stereo microscope with a MicroPublisher CCD cooled color camera and Image Pro Plus v5.0 software.

Results

Molecular characterization of HKL1 knockout and overexpression lines

To understand the biological role of AtHKL1, a functional genomics approach was used by examining phenotypes of transgenic and mutant lines with altered HKL1 protein expression. Seeds of a putative T-DNA insertion line for AtHKL1 were obtained from the Arabidopsis Biological Resource Center (WISCDSLOX383A5). Homozygous knockout plants with a single insert were identified by PCR amplification from genomic DNA using one T-DNA-specific primer and two gene-specific primers. Using the insert specific primer, the T-DNA insertion site was mapped to exon VI of HKL1 (Fig. 3.1 A). Transcript analyses of seedling cDNA using semi-quantitative RT-PCR showed that the mutant line has no detectable HKL1 transcript (Fig.3.1 D) and was thus designated as *hkl1-1*.

Transgenic Arabidopsis plants were made by constitutively expressing HKL1 in

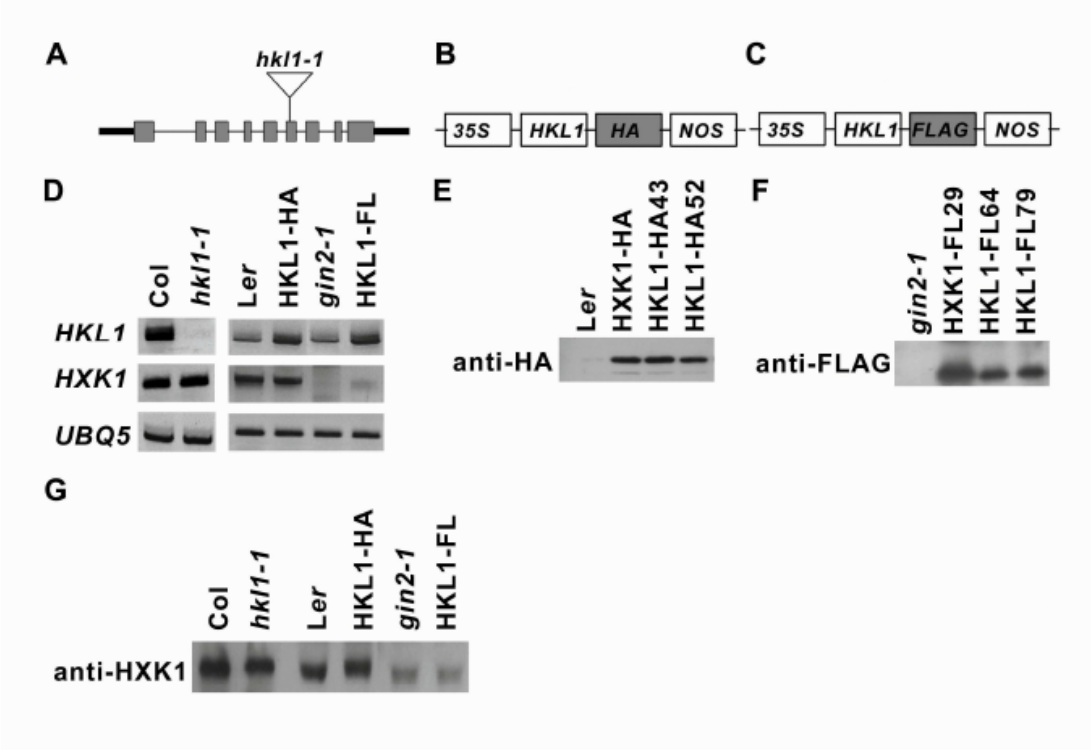

Figure 3.1. Molecular characterization of Arabidopsis HKL1 mutant and transgenic lines. (A) Schematic showing the gene structure of *HKL1*. Exons are indicated by grey rectangles, introns are indicated by lines. The location of the T-DNA insertion in *hkl1-1* is shown with the open triangle. (B,C) Design of plasmid constructs used to transform Arabidopsis lines. HKL1-HA was used to transform Ler and HKL1-FLAG was used to transform *gin2-1*. Boxes are not drawn to scale. 35S = CaMV promoter; NOS = nopaline synthetase terminator. (D) Expression of *HKL1* and *HXK1* transcripts in parental lines and the corresponding mutant or transgenic lines: Col and *hkl1-1*; Ler and HKL1-HA overexpression line 52; and, *gin2-1* and HKL1-FLAG overexpression line 79. *UBQ5* mRNA was used as a control for amount of template. PCR cycle numbers for HKL1, HXK1 and UBQ 5 were, respectively, 33, 30 and 30. (E) Immunoblot analysis of 1 μg protein from leaf extracts of two HKL1-HA overexpression lines, using anti-HA antibody. Shown also is extracted protein from an HXK1-S177A-HA transgenic line, used as a positive control. (F) Immunoblot analysis of 1 μg protein from leaf extracts of two HKL1-FLAG overexpression lines, using anti-FLAG M2 antibody. Shown as well is extracted protein from HXK1-FLAG line 29 (Moore et al. 2003), also expressed in the *gin2-1* background and used herein as a positive control.

different genetic backgrounds either in Ler as HKL1-HA or in *gin2-1* as HKL1-FLAG (Fig. 3.1 B, C). Three independent homozygous lines were obtained for HKL1-HA transformants and seven lines for HKL1-FLAG transformants. The HKL1 mRNA level in the transgenic lines was assessed by semi-quantitative RT-PCR. Both HKL1-HA and HKL1-FLAG transformants had substantially increased HKL1 transcript relative to their respective parental lines (Fig. 3.1 D). Notably, the HXK1 mRNA abundance was not altered in *hkl1-1* or in transgenic lines expressing HKL1-HA (Fig. 3.1 D). In contrast, the HKL1-FLAG lines did not have any detectable HXK1 transcript (Fig. 3.1 D), consistent with their parental background being *gin2-1*.

Western blot analysis of leaf extracts was carried out using either antibodies to the introduced epitope tag or a polyclonal antibody to AtHXK1 (Moore et al. 2003). All of the transgenic lines expressed the corresponding tagged protein, while the parental lines did not (Fig. 3.1 E, F). Positive controls included available transgenic lines that expressed either HA- or FLAG-tagged variants of HXK1 protein. From these assays, two indicated lines expressing each construct were selected for further phenotypic analyses, with data presented for HKL1-HA line 52 and for HKL1-FLAG line 79.

Growth phenotypes of HKL1 knockout and overexpression lines

To test whether the HKL1 protein has a discernable function in plant growth, corresponding parental, transgenic, and mutant lines were grown under different conditions. When grown on 1X MS agar plates with 0.5% sucrose, *gin2-1* seedlings were

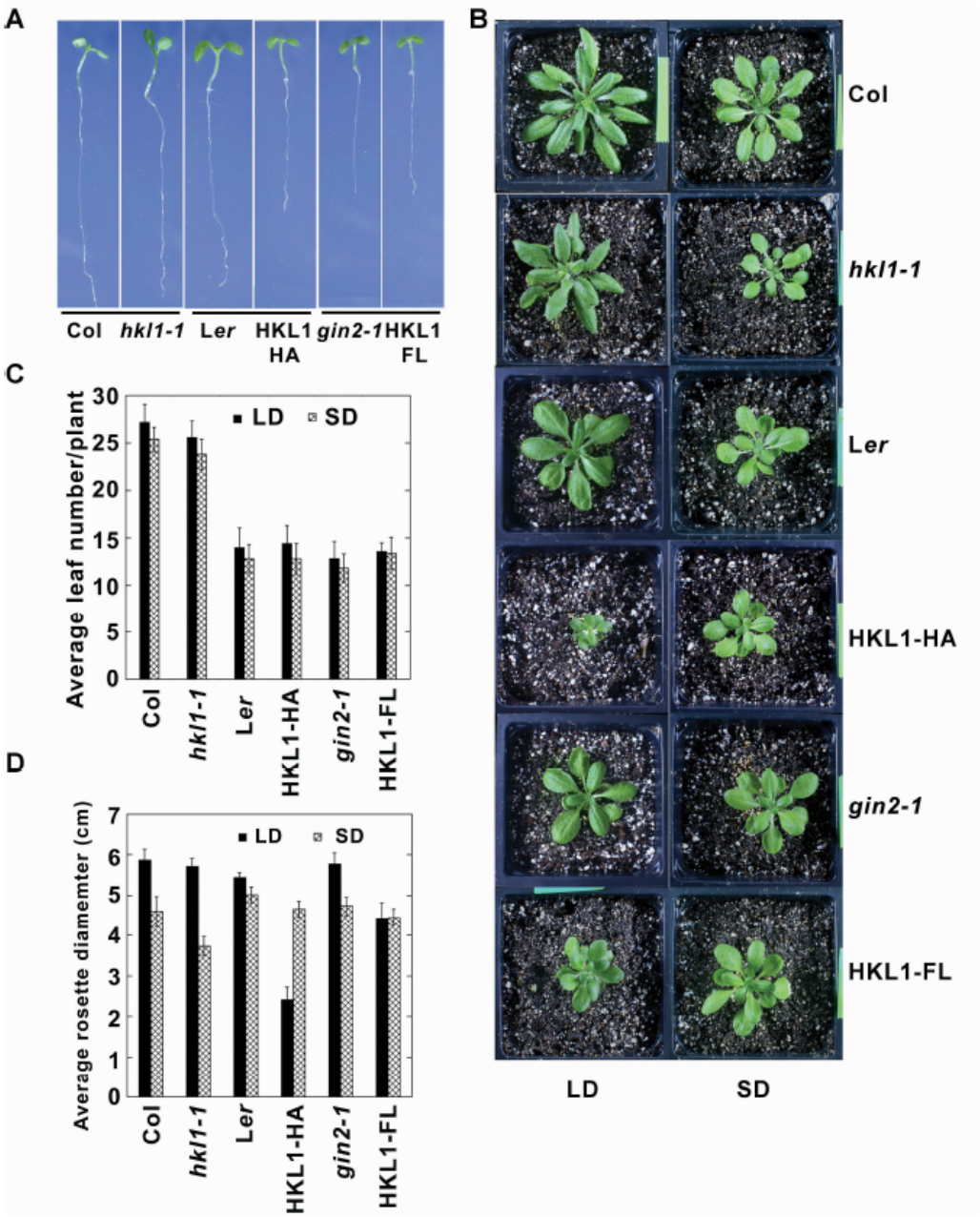

Figure 3.2 Growth phenotypes of HKL1 mutant and transgenic lines. (A) Seven d old seedlings from the different lines on 1X MS plates + 0.5% Suc. (B) Plants after 30 d in a growth chamber under 8 h (SD) or 16 h (LD) photoperiods. (C) Leaf number per plant, as influenced by photoperiod. (D) Average rosette diameter, as influenced by photoperiod. Values for (C) and (D) are means ± 1 SD (*n*=10).

somewhat smaller in size compared to Ler (Fig. 3.2 A), as previously observed (Moore et al. 2003). Interestingly, HKL1-HA seedlings were also smaller than Ler and resembled *gin2-1* seedlings on sucrose plates. However, overexpression of HKL1 in the *gin2-1* background had little affect on seedling growth. Also, growth of *hkl1-1* seedlings resembled growth of the control Col seedlings. These results indicate that HKL1 might be a negative regulator of plant growth, but only if HXK1 is present.

Transgenic and mutant lines also were grown in soil and examined for possible differences in their response to photoperiod or light intensity. When grown under SD conditions, both HKL1 overexpression lines had no substantial growth differences, when compared to control plants (Fig. 3.2 B, D). However, under SD conditions the *hkl1-1* plants were somewhat smaller than control plants, with rosettes about 20% smaller in diameter (Fig. 3.2 B, D). Under LD conditions, the growth of both overexpression lines was notably reduced. The rosette diameter of HKL1- HA plants was 50% less than that of Ler plants, and the diameter of HKL1-FLAG plants was reduced by 25% compared to *gin2-1* plants. However, under LD conditions growth of the *hkl1-1* plants was similar to controls. The observed reductions in rosette sizes were not associated with a change in leaf numbers among the different genotypes at the time of flowering (Fig. 3.2 C). Also, the transgenic and mutant lines did not show any significant change in time to flowering under either photoperiod (data not shown). The latter indicates that the overall developmental program has not changed due to changes in HKL1 protein expression. Notably, while the HKL1 overexpression phenotypes depend largely on the presence of HXK1, they do not always have the same characteristics as those of *gin2-1*.

Modulation of HKL1 expression affects elongation of seedling hypocotyls

Seedling hypocotyl growth is highly influenced by diverse factors that regulate cell elongation in plants, including light, hormones and temperature. For our hypocotyl growth assay, HKL1 transgenic and mutant seedlings were grown vertically on agar plates under constant low light conditions and hypocotyl lengths were measured after 7 d. The average hypocotyl length of *gin2-1* seedlings was about 45% less than for Ler seedlings (Fig. 3.3). Interestingly, the hypocotyl length of HKL1-HA seedlings was about 50% shorter than for Ler seedlings (Fig. 3.3). On the other hand, *hkl1-1* seedlings

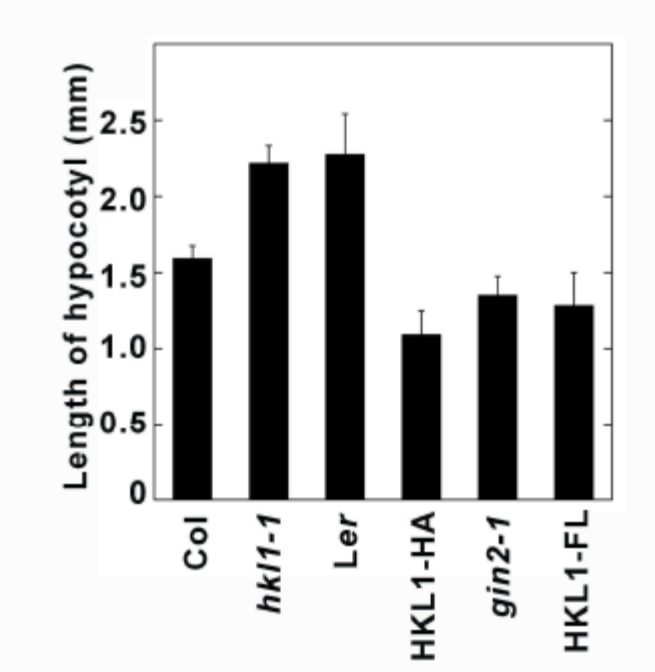

Figure. 3.3 Seedling hypocotyl growth of different HKL1 expression lines. Seedlings were grown for 7 d on 1/5X MS plates under constant light (15 µmol m^{-2} s^{-1}) at 22 ^{o}C. Values are mean hypocotyl lengths ± 1 SD (*n*=15).

had a 40% increase in hypocotyl length relative to Col seedlings (Fig. 3.3). However, HKL1-FLAG seedlings did not show any significant change in hypocotyl growth when compared with the parental genotype, *gin2-1*. These data indicate that HKL1 acts as a negative regulator of seedling hypocotyl growth, but only if HXK1 is present.

HKL1-HA is insensitive to auxin induced lateral root initiation

The reduced hypocotyl elongation of *gin2-1* seedlings was previously linked to its being relatively insensitive to auxin (Moore et al. 2003). Since HKL1-HA seedlings

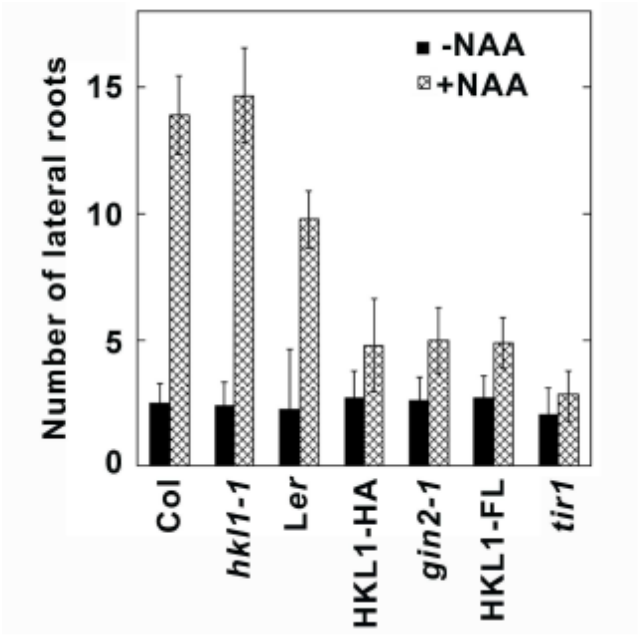

Figure 3.4 Auxin induced lateral root formation in seedlings of different HKL1 expression lines. The number of lateral roots were measured 5 d after transfer from plates with 5 µM NPA to plates ± 0.1µM NAA (see Materials and methods). Values are average lateral root numbers ± 1 SD (*n*=10).

resembled *gin2-1* in the hypocotyl growth assay, transgenic and mutant lines were also characterized by an auxin response assay. In this assay, seedling growth in the presence of the auxin transport inhibitor NPA greatly reduces the number of lateral roots (Himanan et al. 2002). Lateral root formation then can be initiated after seedling transfer to plates with auxin. By this assay, both Col and Ler seedlings showed a robust auxin induction of lateral root formation, increasing 5-fold and 4-fold, respectively, after transfer to plates with NAA (Fig. 3.4). Seedlings of *hkl1-1* showed a similar increase in the number of lateral roots relative to Col seedlings. However, auxin treatment induced relatively few lateral roots in *gin2-1*, HKL1-HA and HKL1-FLAG seedlings (about 2- fold). As a control for this assay, auxin treatment of the receptor mutant *tir1* did not result in appreciable lateral root formation. These data indicate that the HKL1 protein acts as a negative regulator of auxin-induced lateral root formation, again depending on the presence of HXK1.

HKL1 mutants and transgenics have altered glc dependent phenotypes

The described growth phenotypes of the HKL1 transgenic and mutant lines in principle could be due to an influence of HKL1 protein on HXK1 protein catalytic activity, signaling function, or neither. To test for the possible influence of HKL1 protein on glc phosphorylation activity, rate measurements were carried out using leaf extracts from the different lines. There was no significant difference for enzyme activity between

the transgenic lines and their respective parental lines (Fig. 3.5 A). As reported previously (Moore et al. 2003), HXK enzyme activity in *gin2-1* is about(Moore et al. 2003), HXK enzyme activity in *gin2-1* is about half of that in Ler. These results support the Western analyses (Fig. 3.1 G), in which HXK1 protein levels were comparable among corresponding lines. The possible inhibition of HXK1 by HKL1 was also tested

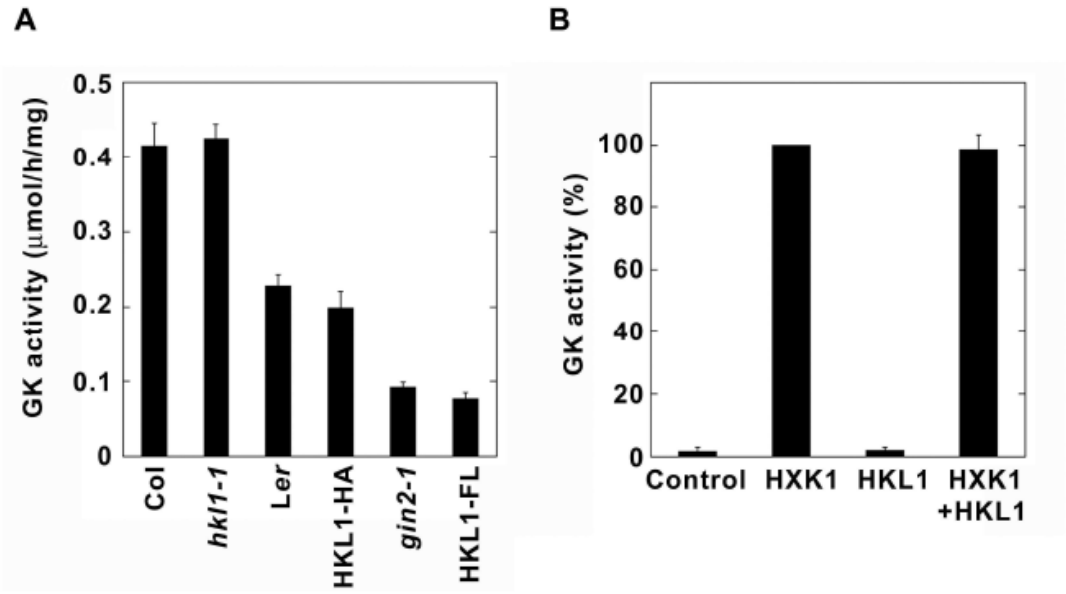

Figure 3.5 Glucokinase activity of different HKL1 expression lines. (A) Clarified leaf extracts of greenhouse grown plants were assayed directly for enzyme activity. Values are means ± 1 SD (*n*=3 biological replicates). (B) Maize protoplast extracts were assayed directly for enzyme activity after expression of HXK1-HA and/or HKL1-HA cDNAs. Protein expression was routinely monitored by labeling with [^{35}S]-methionine as previously described for these constructs (Karve *et al*., 2008). Values are means ± 1 SD from 3 separate transfections, expressed relative to control, untransfected protoplasts.

directly by transiently expressing HXK1-HA and HKL1-HA in maize protoplasts. HKL1-HA did not have any glc phosphorylation activity, as previously reported (Karve et al. 2008). When co-transfected with HXK1-HA, HKL1 protein did not affect

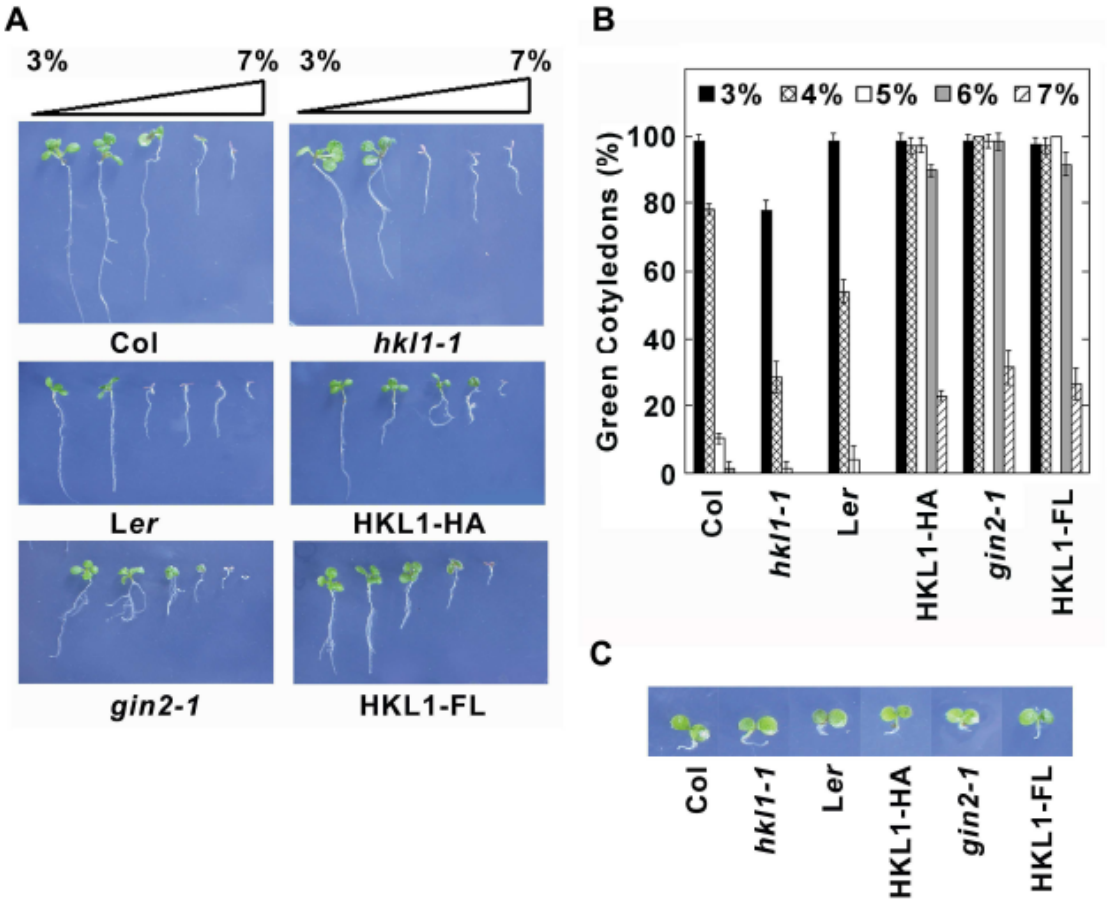

Figure 3.6. Phenotypes of different HKL1 expression lines grown on glc plates. (A) Images are of representative seedlings grown 7 d on 1X MS plates with varying concentrations of glc (3-7%). (B) Percentage of seedlings from plates in (A) that had green cotyledons. Values are expressed relative to the total number of germinated seedlings and are means ± 1 SD (*n*=3 plate replicates). (C) Images of representative 7 d old seedlings grown on 1X MS plates with 6% mannitol.

measured glucokinase activity (Fig. 3.5 B).

Since HKL1 lacks glucokinase activity despite having a largely conserved glc binding domain, it is possible that the protein affects glc or other unknown cellular signaling activities. A widely used phenotypic screen to identify mutants in sugar

signaling is based on the ability of some mutants to develop normally on otherwise inhibitory concentrations of exogenous glc (Rolland et al. 2006). Therefore, seedling growth of the different lines was assessed in the presence of varying glc concentrations (Fig. 3.6 A, B). At relatively high glc levels, Col and Ler seedlings underwent developmental arrest, with much reduced root and shoot growth, and did not accumulate chlorophyll. The *hkl1-1* seedlings were hypersensitive to developmental arrest, showing substantial repression even at 4% glc. In contrast, the HKL1-HA seedlings were glc insensitive relative to the Ler parental line, with >90% of seedlings having green cotyledons when grown on 6% glc. The responses of HKL1-FLAG seedlings were comparable to those of *gin2-1* seedlings, but were not synergistic (data not shown). When grown instead on MS plates with 6% mannitol, all lines had similar growth, albeit with somewhat smaller roots (Fig. 3.6 C). Therefore, the observed developmental arrest on glc plates is not a result of osmotic stress. The glc hypersensitive phenotype of the *hkl1-1* mutant along with the glc insensitive phenotype of the HKL1-HA line indicates that HKL1 could be a negative regulator of glc signaling.

HKL1 protein might not regulate glc responsive promoters

To test whether HKL1 might affect the expression of glc responsive genes, protoplast transient expression assays were done using p*RBCS*-LUC and p*ASN1*-LUC as established reporters of HXK1 signaling (Balasubramanian et al. 2007). LUC activities were normalized for possible variations in transfection efficiency by including a control

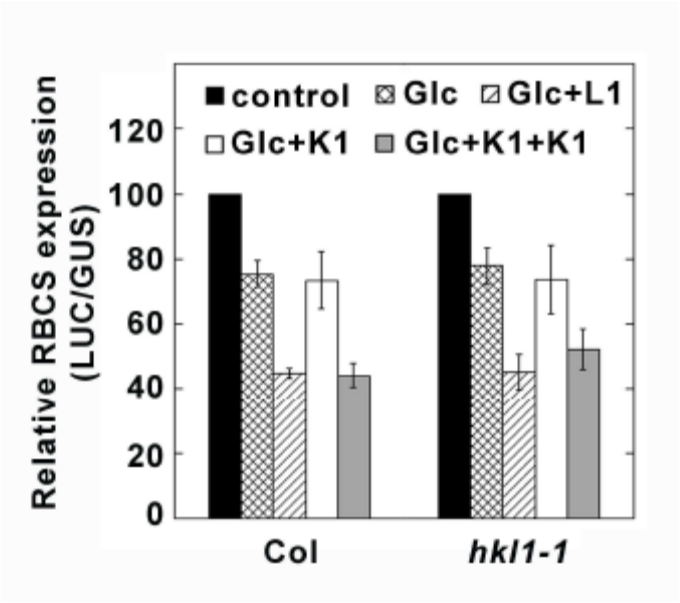

Figure 3.7 Protoplast signaling assay using WT Col or *hkl1-1*. Arabidopsis leaf mesophyll protoplasts were co-transfected with *RBCS-LUC* and *UBQ10-GUS*, plus or minus effectors HXK1-HA and/or HKL1-HA. Protoplast conditions are without glc (control), with 2 mM glc, with 2 mM glc + HXK1-HA (Glc + K1), with 2 mM glc + HKL1-HA (Glc+ L1), and with 2 mM glc + HXK1-HA + HKL1-HA (Glc + K1+ L1). Values are means of 3 replicate assays ± 1 SD, after normalizing the relative LUC units with respect to GUS activity. GUS activity was not affected by the presence of glc or either effector.

promoter construct p*UBQ10*-GUS. Leaf protoplasts of Col and *hkl1-1* plants were used in independent assays. Relative RBCS-LUC activity in both protoplast types was reduced by 25% with 2 mM glc. In both cases, co-transfection with HXK1 plus treatment with 2 mM glc reduced the reporter activity by about 55% (Fig. 3.7). In contrast, transfected HKL1 did not affect the relative RBCS-LUC activity with glc alone or with HXK1 plus glc, using protoplasts from either genotype. Similar results were obtained for the relative activity of ASN1-LUC in the wt Col protoplasts (data not shown). Notably, in all cases the expression of UBQ10-GUS was not affected by co-transfection of HXK1, HKL1 and/or by addition of 2 mM glc. The lack of any defect in

glc repression of expressed RBCS-LUC in *hkl1-1* protoplasts, plus the lack of response by the *RBCS* and *ASN* promoters to HKL1 transfection, both indicate that AtHKL1 likely does not directly affect HXK1-dependent glc signaling.

HKL1 promoter directs tissue specific GUS expression and is regulated by plant hormones

As an alternative approach to characterize HKL1 function, transgenic Arabidopsis plants were made that express an *HKL1* promoter-GUS fusion construct (pHKL1-GUS). Three independent homozygous transgenic lines were then examined, first by histochemical GUS staining. At early stages of seedling development, GUS activity was detected mainly in the root, particularly towards the root tip (Fig. 3.8A). With increased seedling growth, staining was progressively localized to the vascular tissues of roots and cotyledons (Fig. 3.8 B). GUS activity was relatively strong in the root and shoot meristems (Fig. 3.8 C), but not in leaf primordia. In adult plants, GUS expression was highest in the root and leaf vascular tissue and in the emerging lateral roots (Fig. 3.8 D, E, F). In stem cross-sections, GUS staining was observed in the phloem. In flowers, GUS staining was observed in the anther filaments, but not in the pistils (Fig. 3.8 G, H). Staining was also observed in the vasculature of developing siliques, localizing to the funiculi of developing seeds (Fig. 3.8 I). Since HKL1 overexpression reduced the sensitivity of seedlings to auxin dependent lateral root formation (Fig. 3.4) and reduced

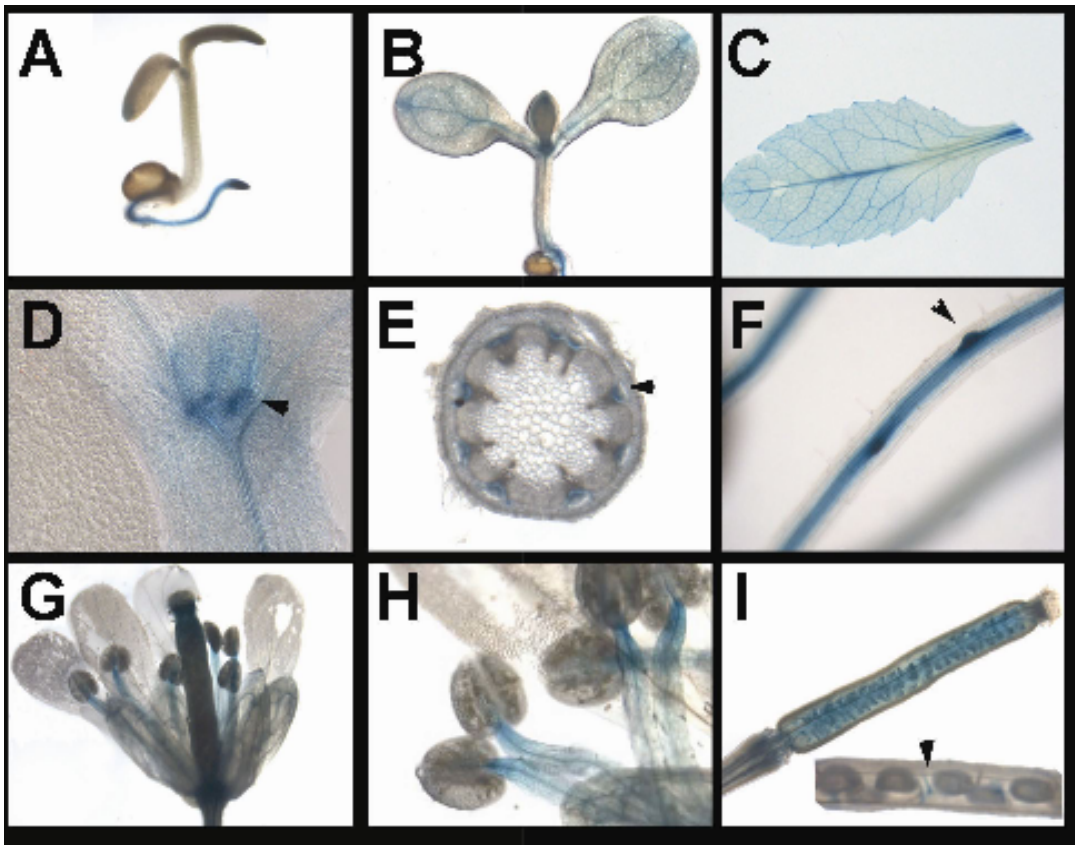

Figure 3.8 Organ and tissue expression of *pHKL1-GUS*. (A) Seedlings grown 3 d on 1X MS plates. (B) Seedlings grown 7 d. (C) Leaf from a 21 d old plant. (D) Shoot of a 5 d old seedling, with arrow pointing to specific staining in the meristem. (E) Stem cross section, with arrow pointing to phloem. (F) Root of a 10 d old seedling. Note staining at the site of lateral root initiation. (G) Opened flower. (H) Anthers and filaments. (I) Developing silique, with insert showing a mature silique and arrow pointing to the funiculus of a developing seed.

the sensitivity of seedlings to glc repression of development (Fig. 3.6), the influence of several different hormones on the expression of pHKL1-GUS fusion was examined in the transgenic seedlings. The seedlings were grown on agar plates for 7 d, and then transferred for 4 h to liquid medium containing indicated hormone additions (Fig. 3.9). The effect of the hormone treatments was examined visually by GUS staining

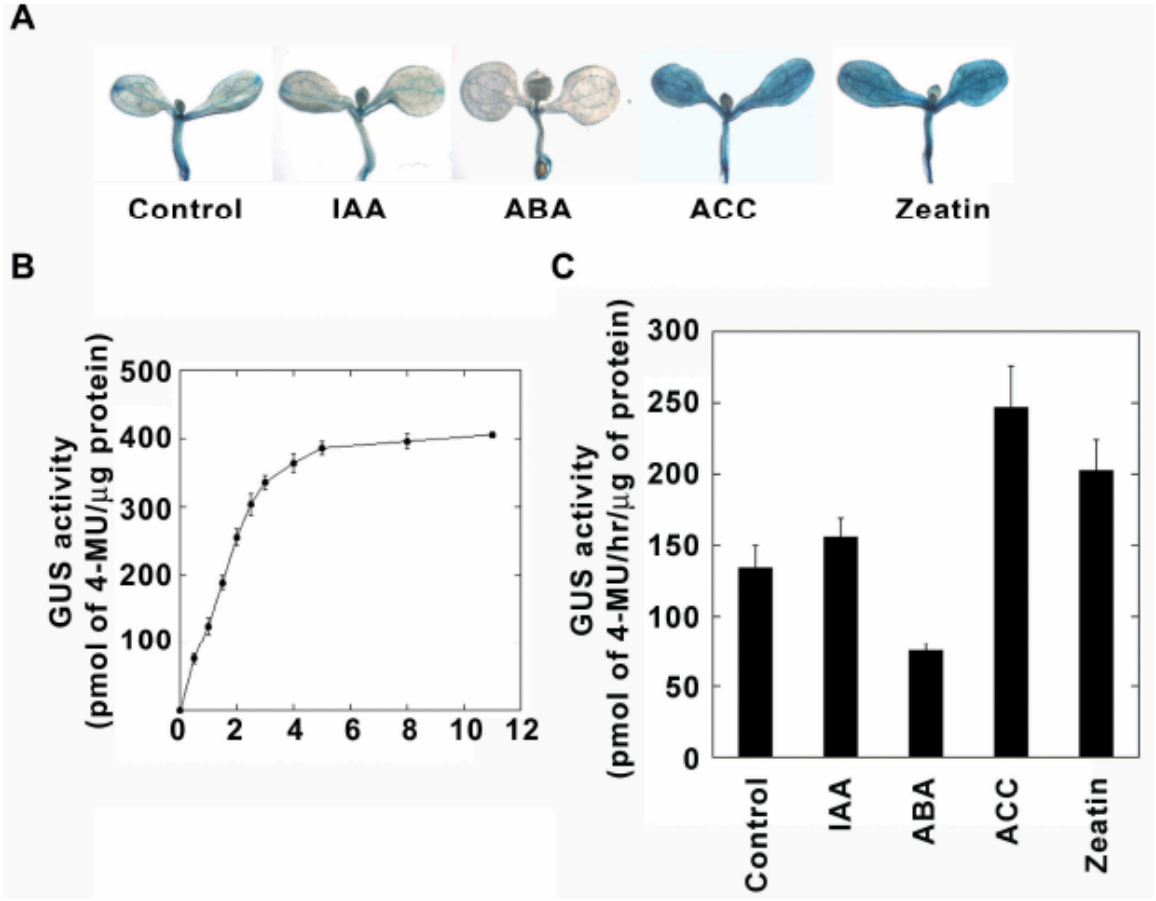

Figure 3.9 Effect of different plant hormones on *pHKL1-GUS* expression. Seedlings of pHKL1-GUS lines were grown 7 d on 1X MS plates, then transferred to liquid MS medium for 4 h with different plant hormones: 10 µM IAA, 1 µM ABA, 50 µM ACC, and 10 µM zeatin. (a) Seedlings stained for GUS activity. (b) Time course for the activity assay after extraction of control seedlings. (c) GUS activity over a 2 h assay following extraction of treated seedlings. Values are means of 3 independent samples ± 1 SD.

and quantitatively after extraction and direct assay of GUS activity. Initial assays in the absence of added stimuli established that a 2 h reaction time with seedling extracts was within the linear range of activity.

IAA or GA3 treatments did not induce pHKL1-GUS expression or activity (Fig. 3.9 A, C; GA3 treatment not shown). However, ABA treatment greatly reduced seedling

GUS staining and reduced the extractable GUS activity by 50% (Fig. 3.9 A, C). On the other hand, zeatin or ACC treatments induced GUS expression throughout the seedling and not just in the vascular tissues (Fig. 3.9 A). Correspondingly, the extracted GUS activities following those treatments was up to 2-fold higher than in the control seedlings (Fig. 3.9 C). The results of the GUS assays indicate that the HKL1 gene is regulated by multiple plant hormones and could play a crucial role in plant growth and development.

Discussion

In Arabidopsis, HXKs exist as a family of six genes. As an enzyme catalyst, HXK1 is required for glc phosphorylation. As a glc sensor protein, HXK1 is required for normal plant growth and development, in part by influencing other hormone signaling pathways (Moore et al. 2003). Much of the related previous research on glc signaling by HXK1-dependent processes have focused on identifying downstream components and mechanisms (*e.g.*, Ho et al.. 2001; Yanasigawa et al. 2003; Cho et al. 2006b; Balasubramanian et al. 2007). However, AtHXK2 also functions as a glc sensor/transducer (Jang et al. 2007), and other members of the gene family, including some of the non-catalytic proteins, might have related roles as well.

Elucidating functions of non-catalytic proteins can be challenging. Examining the tissue expression pattern of a gene can provide clues for function. HKL1 transcript was previously shown to be expressed in principal plant organs (Karve et al. 2008). These observations were extended in this study by demonstrating that pHKL1-GUS activity

occurs predominantly in vascular tissues of roots, stems, young leaves, stamen filaments and developing embryos (Fig. 3.8). In stem cross-sections, staining was most apparent in phloem tissue. HKL1 expression also was strong in root tips and in lateral root primordia. While we are not aware of any HXK family members having been reported in surveys of the phloem proteome, nonetheless, many phytohormones and a number of signaling proteins have been detected in phloem sap (*e.g.*, Giavalisco et al. 2006).

Phenotypes of AtHKL1 knockout mutant and overexpression lines provide evidence that a major function of the HKL1 protein is to modulate plant growth. HKL1 overexpression in Ler (HKL1-HA) resulted in several distinct phenotypes, including reduced seedling growth on sucrose plates (Fig.3 2), reduced hypocotyl elongation under low light conditions (Fig. 3.3), severe reduction in rosette size under LD conditions (Fig. 3.2), and seedling growth tolerance on agar plates with 6% glc (Fig. 3.6). The HKL1 knockout line, *hkl1-1*, did not show significant growth differences from the WT on sucrose plates, but mutant plants did have contrasting phenotypes to the overexpression lines by having increased hypocotyl growth at low light, reduced rosette size under SD conditions, and hypersensitivity to growth on agar plates with glc (Figs. 3.2, 3, 6). Many of the phenotypes of HKL1-HA transgenic plants are similar to those previously reported for the HXK1 null mutant, *gin2-1* (Moore et al. 2003). These phenotypes indicate that HKL1 is a negative regulator of plant growth. In an initial recent report, some rice HXK family members also are noted as being apparent negative regulators of seedling growth (Yu and Chiang, Plant Biology 2008).

Interestingly, phenotypes of HKL1 overexpression lines in the *gin2-1* background were generally the same as the parental line. That is, HXK1 protein was required for most of the examined phenotypes associated with altered expression of HKL1 protein. Activity assays, Western blots, and analysis of HXK1 mRNA levels (Fig. 3.1, 5) rule out the possibility that HKL1 overexpression in the transgenic lines might somehow have decreased the expression of HXK1 protein. Also, even when HKL1 is transiently expressed at relatively high amounts in protoplasts, accumulated protein did not affect either glc phosphorylation or glc signaling activities of co-transfected HXK1 (Figs. 3.5, 7). Therefore the observed phenotypes for the HKL1-HA lines could be associated with an "indirect" signaling function of HXK1 that might involve other hormones. For example, since mutants of Arabidopsis with constitutive ethylene signaling activity are tolerant to growth on plates with 6% glc (Zhou et al.1998), HKL1 overexpression could somehow promote ethylene signaling or tissue sensitivity. Since both HXK1 and HKL1 are targeted to mitochondria (Heazelwood et al. 2004; Karve et al. 2008), the two proteins might interact and thereby influence a number of related growth processes. On the other hand, there are some different phenotypes among lines with altered expression of HXK1 and HKL1 proteins. In contrast to the influence of photoperiod on growth of HKL1-HA and *hkl1-1* lines, plant growth of *gin2-1* was minimally affected by photoperiod (Fig. 3.2). Also, while growth of *gin2-1* plants is repressed at relatively high light intensity (Moore et al. 2003), the different HKL1 expression lines do not show reduced growth under the same high light conditions (data not shown). Thus, these data

suggest that functions of HXK1 and HKL1 might converge downstream of the primary sensor protein HXK1.

Seedling hypocotyl growth by cell elongation is strongly influenced by many factors including light, temperature, nutrients, and plant hormones such as auxin, cytokinin, ethylene, gibberellins and brassinosterioids (Collet et al. 2000; Vandenbussche et al. 2005). In *gin2-1*, reduced hypocotyl growth has been attributed to its possible insensitivity to auxin signaling (Moore et al. 2003). Similar to *gin2-1*, HKL1-HA seedlings also have shorter hypocotyls (Fig. 3.3). Since *hkl1-1* seedlings have longer hypocotyls, we speculate that the HKL1 protein might be a negative regulator of cell expansion. Interestingly, ethylene also can repress hypocotyl elongation in seedlings grown under conditions similar to those in our experiment (Smalle et al., 1997). Therefore, altered seedling responses to either ethylene or auxin might account for the observed HKL1-dependent hypocotyl growth phenotypes.

Since HKL1-GUS activity was observed at sites of lateral root formation, we also examined related transgenics for their sensitivity to auxin-induced lateral root formation. As with other phenotypes described above, the HKL1 protein is a negative regulator of root formation, HXK1 is a positive regulator, and the HKL1 phenotype is not additive in the absence of HXK1 (Fig. 3.4). While lateral root formation does require auxin synthesis, transport, and/or signaling, enhanced ethylene signaling has recently been shown to repress lateral root formation by modulating auxin transport (Negi et al. 2008). Thus, this observed HKL1 phenotype also might be associated with enhanced ethylene signaling, as suggested above for plant growth under LD conditions.

In addition to the possible role of HKL in mediating cross-talk with some phytohormone dependent processes, promoter expression analysis suggests a further possible inter-connection between hormone signaling and HKL1 functions. The HKL1 promoter is repressed by ABA and induced by both ACC and cytokinin, but IAA treatment had little effect. The ABA repression is consistent with the repression of HKL1 transcript by salt and osmotic stress (Claeyssen and Rivoal, 2007), processes in part regulated by ABA. The HKL1 promoter does have two ABA response elements and one ethylene response element (Molina and Grotward 2005; Obayashi et al. 2007). Further analysis is needed to confirm the role of these elements. However, the HXK1 and HKL1 promoter sequences are not likely to be co-regulated (Weeder Web interface; Pavesi et al. 2004), consistent with the premise that these proteins do have some different affects on plant growth.

Non-catalytic HXKs have been experimentally identified as regulatory proteins in fungi including *S. cerevisiae* and *A. nidulans* (Daniel 2005; Bernardo et al. 2007). The extent to which non-catalytic homologs are present in other protein families with known enzymes is not clear. The Arabidopsis glutathione transferase family does include both non-catalytic as well as catalytic forms, though their relative distribution between groups apparently has not been strictly determined (Dixon et al. 2003). Recently, β-amylase4 (BAM4) of Arabidopsis was shown to lack apparent catalytic activity, yet to somehow facilitate starch breakdown (Fulton et al. 2008). BAM4 is one of perhaps four chloroplastic isoforms within Arabidopsis. Protein annotations from genome sequencing projects are often done according to general homology to a protein with known function.

However, estimates are that homologs of catalytic proteins require >75% amino acid sequence identity to transfer all 4 digits of an EC number at an error rate below 10% (Rost et al. 2003). Therefore, we speculate that non-catalytic enzyme homologs might occur relatively often within plant gene families, since sequence divergence levels are often >25%. Among Arabidopsis HXKs, four of the six members show about 50% sequence identity to HXK1, and three of those lack catalytic activity (Karve et al. 2008). In the present study, our results indicate that the non-catalytic HKL1 protein can substantially influence plant growth, likely by somehow influencing ethylene and/or auxin response pathways.

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CHAPTER FOUR

A ROLE OF HKL1 IN MEDIATING CROSS-TALK BETWEEN GLUCOSE AND ETHYLENE SIGNALING

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Abstract

The initial characterization of HKL1 transgenic and mutant lines revealed that HKL1 has a crucial role in plant growth, but perhaps only an indirect role in glucose signaling. We therefore measured the transcripts of known glucose repressed genes in HKL1 transgenics and mutants. Our results show that HKL1 does not affect the expression of these target genes and further establish that HKL1 does not have a direct role in glucose signaling. Since growth phenotypes of plants with altered HKL1 expression might be associated with plant ethylene signaling, we used seedling growth and gene expression assays to test for HKL1 function in ethylene responses. HKL1 was found not to affect the classic seedling triple response, which is mediated by ethylene. However, treatment with ethylene precursor did not overcome the glucose-dependent developmental arrest in *hkl1-1*, in contrast to wild type seedlings. Furthermore, HKL1 is required for ethylene-dependent regulation of some ethylene response genes and for glucose-dependent regulation of some ethylene biosynthesis genes. We also discovered a

conditional root hair phenotype in HKL1 overexpression lines and *gin2-1*, a null mutant of hexokinase1. Seedlings of these lines produced bulbous root hairs upon transfer to agar plates with 6% glucose. In HKL1-HA seedlings, the bulbous root hairs were also observed upon transfer to ACC plates, but not in *gin2-1*. Using known ethylene biosynthesis and response mutants, we showed that the root hair response depends on ethylene biosynthesis. We also found that many of the HKL1 phenotypes require hexokinase1 protein and that both proteins can physically interact. Therefore, we suggest that HKL1 mediates cross-talk between glucose and ethylene signaling.

Introduction

To regulate growth and development, plants need to sense, transmit and respond to internal hormonal signals and external stimuli such as nutrients, light, temperature and biotic and abiotic stresses. In plants, sugars generated through photosynthesis not only act as central energy molecules, but also act as hormone-like signaling molecules that modulate other plant signal transduction pathways such as light, hormone, and nitrogen signaling (Smeekens 2000; Coruzzi and Zhou 2001; Leon and Sheen 2003; Gibson 2004).

Genetic and molecular analyses of sugar response mutants have shown that sugar signaling in plants is closely associated with plant hormone biosynthesis and signaling, particularly with abiscisic acid (ABA) and ethylene (Leon and Sheen 2003). Several ABA deficient mutants such as *abi4* and *aba2* have been identified as sugar insensitive in different phenotypic screens (Dekkers et al. 2008; and references therein). This has led to the suggestion that glucose (glc) signaling in Arabidopsis seedlings requires an intact ABA signaling pathway (Smeekens 2000). In addition, the overexpression of some Arabidopsis ABA-response element binding factors (ABF3 and ABF4) confers both ABA and glc oversensitive phenotypes. This further indicates that prominent interactions do occur between glc and ABA signaling (Kang et al. 2002).

The addition of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) alleviates glc-induced inhibition of seedling development in Arabidopsis (Zhou et al. 1998). Interestingly, both a constitutive ethylene biosynthetic mutant, *eto1*, and a

constitutive ethylene signaling mutant, *ctr1*, are insensitive to glc-induced seedling developmental arrest. In contrast, ethylene insensitive mutants such as *etr1, ein2, ein3* and *ein6* exhibit glc hypersensitivity (Zhou et al. 1998). These responses indicate that an antagonistic interaction occurs between glc and ethylene signaling. The cross talk between ethylene and glc signaling is also evident from a DNA microarray analysis following a short term treatment of Arabidopsis seedlings with glc (Price et al. 2004). This study found that several ethylene biosynthesis and signaling genes are repressed by glc, including *CTR1* and genes associated with ethylene metabolism.

Arabidopsis hexokinase1 (HXK1) is probably the best characterized glc sensor in plants and modulates plant growth at different developmental stages, in part through interaction with other hormone signaling pathways (Moore et al. 2003). The Arabidopsis genome encodes three hexokinase (*HXK1, HXK2* and *HXK3*) and three hexokinase-like (*HKL1, HKL2* and *HKL3*) genes. Out of the three HKLs, HKL1 and HKL2 are ~50% identical to HXK1 and lack any detectable catalytic activity (Karve et al. 2008). Both HKL1 and HKL2 have similar tissue expression and sub-cellular localization as does HXK1 and have mostly conserved glc binding domain. These expression features and structural characteristics indicate that HKL1 and HKL2 might have a glc-dependent, regulatory role in Arabidopsis.

Our initial characterization of HKL1 transgenic and mutant lines revealed that HKL1 has a crucial role in plant growth, but apparently only an indirect role in glc signaling (Chapter 3). Changing the amount of HKL1 expression affected seedling and hypocotyl growth, plant growth under different photoperiods, seedling sensitivity to glc

induced developmental arrest, and sensitivity to auxin induced lateral root initiation. Many of the phenotypes of transgenic overexpression line, HKL1-HA in Ler background, were similar to those reported for an HXK1 null mutant, *gin2-1* (Moore et al. 2003), indicating that HKL1 acts as a negative regulator of plant growth. Moreover, overexpression of HKL1 in *gin2-1* did not affect the phenotypic responses of *gin2-1*, indicating that HXK1 protein is required for most of the examined phenotypes associated with HKL1. The observed growth phenotypes of HKL1 indicated that HKL1 influences some phytohormone dependent processes. In addition to the growth phenotypes, a possible interconnection between hormone signaling and HKL1 function was revealed by the observation that the HKL1 promoter is repressed by ABA, but is induced by ethylene and cytokinin.

To understand if HKL1 has a role in mediating cross-talk between glc and ethylene signaling, a candidate gene approach was used. We studied the transcript levels of genes that are known targets of glc and ethylene signaling, as well as genes that are involved in ethylene biosynthesis or signaling, but are known to be regulated by glc. The results of our gene expression analysis show that HKL1 does not have a direct role in glc signaling. However, HKL1 was shown to have a role in mediating glc responses to several genes in ethylene biosynthesis and signaling. In this study, we also have identified a novel ethylene dependent root hair phenotype which is associated with HKL1 expression. Our results reveal novel interactions between glc and ethylene signaling pathways during root hair development and show that HKL1 has a role in this process.

Materials and methods

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col), ecotype Landsberg erecta (Ler), ecotype Wassilewskija (Ws) and *hkl1-1* knockout line in Col background (WISCDSLOX383A5) were obtained from the Arabidopsis Biological Resource Center (Ohio State University); *gin2-1* was as previously described (Moore et al. 2003). The HKL1-HA and HKL1-FLAG homozygous transgenic lines were generated in L*er* and *gin2-1* backgrounds, respectively. As described in the previous chapter. Two independent transgenic lines of each type were used in all the experiments. Seeds of *eto2-1* and *ein2-1* were from Dr. J-C. Jang (Ohio State University) and seeds of *act2-1* were from Dr. Richard Meagher (University of Georgia). Seeds of maize (*Zea mays* L.) were purchased (Line FR922 X FR967, Seed Genetics).

Plant growth conditions

Arabidopsis seeds were surface sterilized and stratified for 2 days at 4 ^{o}C as in Jang et al. (1997) and usually were grown on 1X MS agar plates (modified basal medium with Gamborg vitamins; PhytoTechnology Laboratories) containing 0.7% phytagar (Caisson Laboratories), pH 5.7, under constant light (30 μmol $m^{-2}s^{-1}$). For glc repression assays, seedlings were grown on 1X MS plates supplemented with 6% glc ± 50 μM ACC (Sigma-Aldrich) for 7 d under constant light.

To monitor glc and ethylene dependent regulation of candidate gene expression, seeds were grown in liquid culture. In this case, 15-20 seeds were put into 125 ml flasks containing 50 ml 1/2X MS basal medium supplemented with 1% sucrose (suc). Seedlings were grown on a rotary shaker with constant agitation at 250 rpm under constant light (70 μmol $m^{-2}s^{-1}$) at 22º C for 7 d. Seedlings were washed and incubated with sugar-free 1/2XMS medium for 24 h in the dark with shaking and then were transferred to fresh sugar free medium (control) or supplemented with 2% glc or 50 μM ACC. Seedlings were treated under constant light with shaking for 8 h, and then harvested by quickly drying on filter paper before freezing in liquid N_2.

Root hair development assay

Seedlings were grown vertically for 7 d on 1/2X MS plates with 0.5% suc or 2% glc under constant light (30 μmol $m^{-2}s^{-1}$). Seven day old seedlings were transferred to agar plates with 6% glc +/- 5 μM silver thiosulfate (STS; PhytoTechnology Laboratories), +/- 1 μM aminoethoxyvinylglycine (AVG; Fluka) or with 6% mannitol, or were transferred to respective sugar plates + 5 μM ACC (0.5% suc + ACC, or 2% glc + ACC). In another experiment, *eto2-1* and Col seedlings were transferred from plates with 2% glc to plates with 6, 7 or 8% glc +/- 0.1 μM, 0.25 μM and 1 μM AVG. Seedling root hairs were imaged directly from the agar plates 4-6 d after transfer, using a Nikon SMZ1500 stereo microscope with a MicroPublisher CCD cooled color camera and Image Pro Plus v5.0 software.

RNA isolation and RT- PCR analysis

Total RNA was isolated from whole seedlings using the RNeasy plant kit (Qiagen). One µg of total RNA was converted to cDNA using the Protoscript II RT-PCR kit (New England BioLabs) according to the manufacturer's instructions. The PCR primer sequences for the candidate genes were generated PCR primer sequences were generated using the AtRTPrimer database (Han and Kim 2006): *ACCOX* (ACC oxidase family protein, At5g58660; 5'-TTA ACC ATG CTC GAA GGT TTC AAC G-3', 5'-CAT CGC TTA TGG CCT GCA TCA TAT C-3';expected size = 466 bp); *ACCS* (ACC synthetase family protein, At5g26740; 5'-TGC TTT CCG CCT TTA ACA CTG GAC-3', 5'-AAC ATT GGC ACC AGC ATA CTC CTT G-3'; expected size = 468 bp); *PEROX* (peroxidase, At1g49570; 5'-TCC GTG GAT TCG AAG TCA TTG AAG-3', 5'- GCA GCC GTA GGA TCT GTC ATT AGG G-3'; expected size = 556 bp); *SP2* (sodium symporter family protein, At4g22840; 5'-GGA TCG AGG AAT TTG TGG CGT AGA-3', 5'-GGT GCG ATT ACA ACC TGA AGA ATG C-3'; expected size = 625 bp); *LRR* (leucine-rich repeat protein kinase, At2g26730; 5'-AAC CGG CGG GAG TAG CTA CAA GGA-3', 5'-CCT CTC GCT GCA GTT ATC GCT ATT C-3'; expected size = 496 bp); *ASN1* (asparagine synthetase1, At3g47340; 5'-TGA TTC TCA GGC CAA GAG AGT TCG T-3', 5'-CCC AAC CAA TGT AGA GCG AAG TGA C-3'; expected size = 413 bp); *T6PSY* (trehalose-6 phospahte synthetase, At1g61800; 5'-GGC AAA ATG CGG TTT CTC AAG TCG-3', 5'- TCC TCC CCG GCT ACT CTT TTG TTA G-3'; expected

size = 626 bp), *GLYK* (glycerate kinase, At1g80380; 5'-TTG GTG CGA AGA TCA GAT TGC TTT G-3', 5'-GGA GAC AGC ATC GCA TTA GTT TGC- 3'; expected size = 544 bp) and *UBQ5* (ubiquitin5, At3g62250; 5'- GTG GTG CTA AGA AGA GGA AGA-3', 5'- TCA AGC TTC AAC TCC TTC TTT-3'; expected size = 254 bp). All the primers were designed to span one or more introns such that the amplicon size from cDNA is different than from genomic DNA. The template amounts were first titrated to balance the *UBQ5* expression in different samples and corresponding template amounts were used thereafter, while varying PCR cycle numbers.

^{35}S- labeling and co-immunoprecipitation

As previously described (Yoo et al. 2007), protoplasts from greening maize leaves were transfected with 6–10 μg of cesium chloride-purified plasmid containing HKL1-GFP, HXK1-HA, and yeast HXK2-HA (YHK2; Moore et al. 2003; Karve et al. 2008). Following transfection, protoplasts were incubated in the dark for 90 min, then [^{35}S] Met (25 μCi; Perkin-Elmer) was added for 8 h. Transfection efficiencies were routinely > 60% as determined using WRKY-GFP (Balasubramanian et al. 2007). Harvested protoplasts were lysed and resuspended as described previously (Balasubramanian et al. 2007). Anti-HA (Roche) or anti-GFP (Sigma-Aldrich) antibodies were used with protein A agarose beads (Roche) for immunoprecipitation. The beads were resuspended in 2X SDS sample buffer, electrophoresed on 10% SDS gels, and visualized by fluorography.

Results

HKL1 does not modulate the expression of known glucose response genes

Our previous results from seedling glc tolerance assays (Fig 3.6) and from protoplast signaling assays (Fig. 3.7) indicated that HKL1 likely does not have a direct role in glc signaling. The potential role of HKL1 in glc signaling was further analyzed by studying the transcript levels of candidate genes in seedlings of HKL1 transgenic and mutant lines grown in liquid culture.

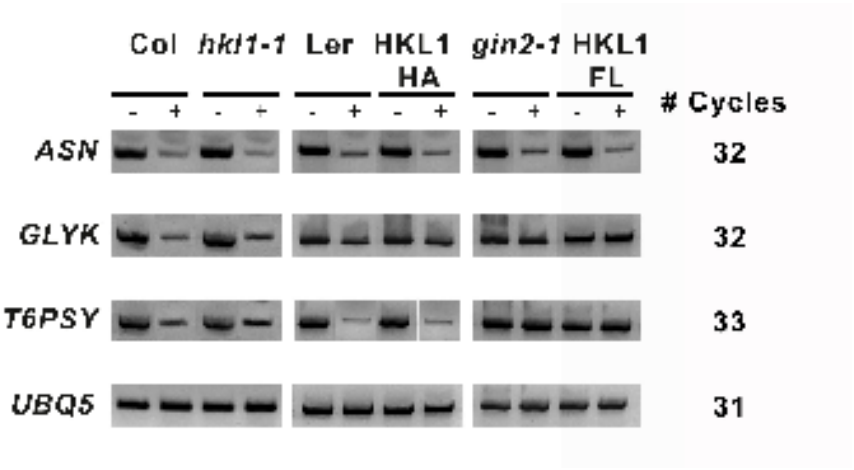

Figure 4.1 Expression of glc regulated genes *ASN, GLYK* and *T6PSY* in HKL1 transgenic and mutant lines. Semi quantitative RT-PCR was used to determine the transcript levels of *ASN, GLYK* and *T6PSY.* The Col, *hkl1-1*, Ler, HKL1-HA, *gin2-1* and HKL1-FLAG seedlings were grown in MS liquid medium as described in Materials and methods with or without 2% glc for 8h. *UBQ5* was amplified as an internal control for the amount of template. Cycle numbers refer to number of PCR reaction to amplify each cDNA

In Arabidopsis, *ASN1, GLYK* and *T6PSY* are transcriptionally repressed by glc (Price et al. 2004). In our assay all three of these were repressed in WT seedlings after glc treatment(Fig. 4.1). In contrast to the response of WT Ler, the transcripts of *GLYK*

and *T6PSY* were not repressed in *gin2-1* seedling after glc treatment. However, the transcript of *ASN1* was repressed after glc treatment in *gin2-1*seedlings. Therefore, *GLYK* and *T6PSY* are regulated by HXK1-dependet glc signaling, where as *ASN1* is regulated by a glc signaling pathway that is independent of HXK1. To test whether HKL1 modulates the expression of these glc response genes, we examined their transcript levels genes in seedlings of the HKL1 transgenic and mutant lines following glc treatment. The transcripts of *ASN1*, *GLYK* and *T6PSY* were repressed by glc treatment in Col, *hkl1-1* and HKL1-HA seedlings (Fig. 4.1). HKL1-FLAG expression in *gin2-1* background did not restore glc sensitivity to either *GLYK* or *T6PSY*. These data extend and support our previous suggestion that HKL1 does not affect glc regulation of HXK1 target genes.

HKL1 might mediate cross talk between ethylene and glucose signaling

Since ethylene and glc can have antagonistic roles in signaling, we tested whether ACC treatment affects glc tolerance or the developmental repression response of seedlings with altered HKL1 expression. For this experiment, seedlings were grown on 6% glc plates, with or without 50 μM ACC. The seedlings of wild type Col and Ler grown only on 6% glc underwent developmental arrest with indicators such as reduced cotyledon expansion and greening (Fig. 4.2). However, when grown on plates with 6% glc + ACC, the growth repression phenotype of the WT seedlings was not observed, as reported previously (Zhou et al. 1998). The *hkl1-1* seedlings also underwent

developmental arrest on 6% glc plates. However, in this case, ACC treatment could not overcome the

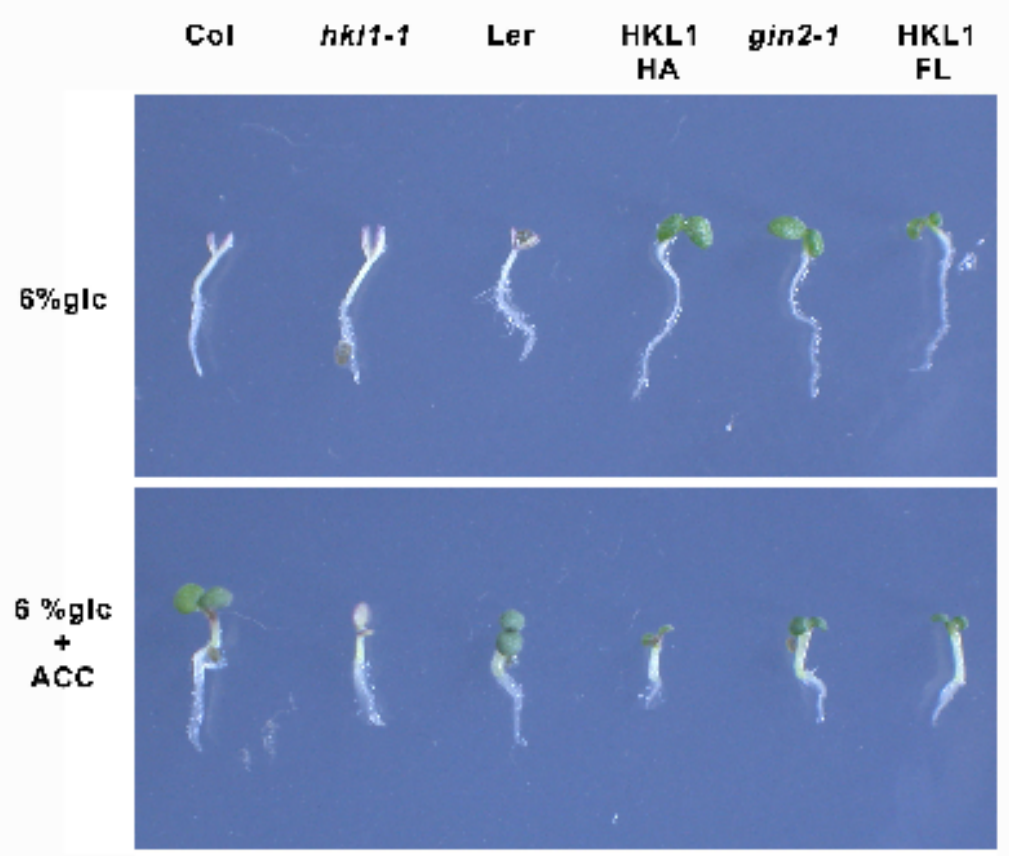

Figure 4.2 Effect of ethylene on glc sensitivity of HKL1 transgenic and mutant lines. Glucose induced developmental arrest was examined in the 7d old seedlings grown on MS medium containing 6% glc, with or without 50μM ACC.

developmental arrest (Fig. 4.2). Seedlings of *gin2-1* and HKL1 overexpression lines (in either background) were insensitive to seedling developmental arrest and produced green cotyledons on 6% glc plates, as observed previously (Fig. 3.6; Moore et al. 2003). ACC treatment did not affect this response but did substantially increase radial expansion of the hypocotyl, while reducing the root length and size of the cotyledons in the HKL1

overexpression lines and in *gin2-1*(Fig. 4.2). These effects were more pronounced in the treated HKL1-HA seedlings. The observed insensitivity of *hkl1-1* seedlings and hypersensitivity of HKL1 overexpression lines to the ACC treatment indicates that HKL1 might mediate cross-talk between some aspects of ethylene and glc signaling, at least during seedling development.

In response to exogenously applied ethylene or its precursor ACC, etiolated WT Arabidopsis seedlings show a characteristic triple response: (1) inhibition of hypocotyl and root elongation, (2) swelling of the hypocotyl, and (3) exaggerated tightening of the apical hook (Guzman and Ecker, 1990). All of our HKL1 transgenic lines and mutant also showed a normal ethylene triple response when grown in the dark in the presence of ACC (data not shown). This indicates that HKL1 expression likely does not affect related responses to ethylene.

HKL1 expression affects expression of some ethylene response and biosynthesis genes

To further define whether HKL1 might have a role in ethylene signaling or how it might affect cross-talk with glc signaling, we examined the transcript levels of two ethylene response marker genes, *SP2* and *LRR* (Nemhauser et al. 2005) in the HKL1 transgenic and mutant lines following short term treatment with ACC or glc (Fig. 4.3 A, B). The *SP2* and *LRR* transcripts were repressed after ACC treatment in Ler, *gin2-1*, and both the HKL1 overexpression lines. While the repression of *SP2* was similar among the different genotypes, the repression of *LRR* was greater in HKL1-HA and HKL1-FLAG seedlings compared to their respective parental lines. ACC treatment also repressed the

transcripts of *SP2* and *LRR* in Col seedlings. However, in *hkl1-1* seedlings, the *SP2* and *LRR* transcripts were not appreciably repressed after ACC treatment. The absence of the

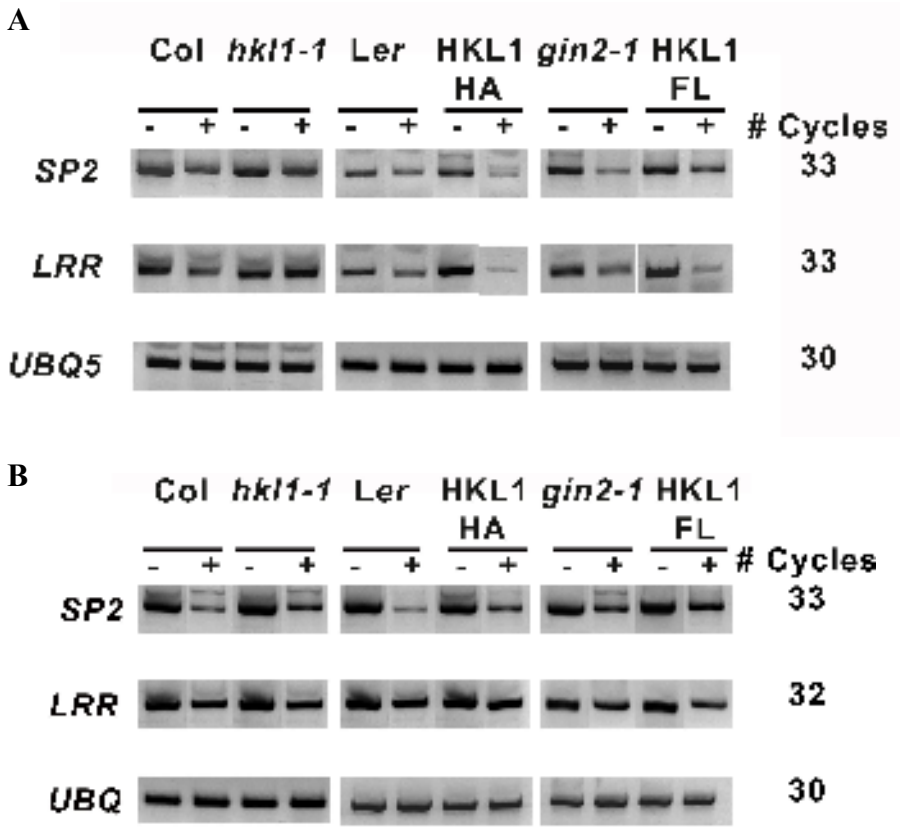

Fig 4.3 Expression of ethylene regulated genes *SP2* and *LRR* in HKL1 transgenic and mutant lines. Semi quantitative RT-PCR was used to determine the transcript levels of *SP1 and LRR* seedlings treated for 8h with 50 μM ACC (A) or 2% glc (B). Seedlings were grown in liquid MS medium as described in Materials and methods with or without 2% glc for 8h. *UBQ5* was amplified as a control for the amount of template. Cycle numbers refer to number of PCR reaction to amplify each cDNA.

gene repression response in *hkl1-1* indicates that HKL1 might be required for ethylene dependent regulation of the *SP2* and *LRR* genes.

On the other hand, treatment with glc repressed *SP2* transcript levels, but not *LRR* transcript levels in all of the genotypes studied (Fig. 4.3B). Glc repression of *SP2* is therefore not dependent on HXK1 or HKL1, and perhaps correspondingly, this repression likely is not antagonistic to ethylene. Notably as well, the role of HKL1 for ACC dependent regulation of *SP2* and *LRR* also does appear to be antagonistic.

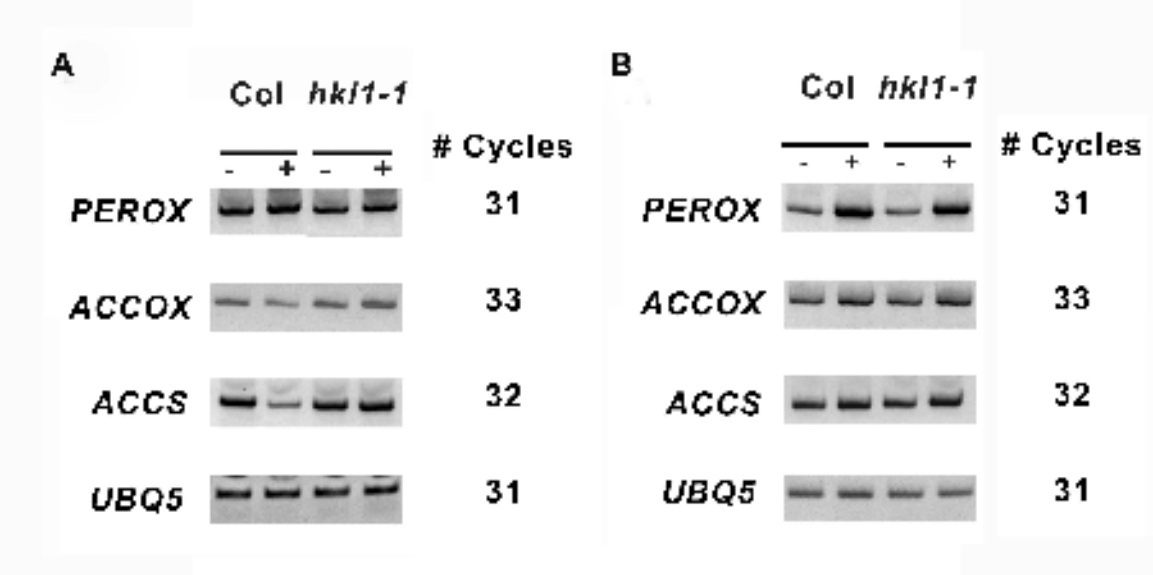

Figure 4.4 The effects of glc and ethylene on the expression of genes associated with ethylene biosynthesis or signal transduction. Semi-quantitative RT-PCR was used to determine the transcript levels of *PEROX, ACCOX* and *ACCOS* from Col and *hkl1-1* seedlings treated for 8h with 2% glc (A) or 50 μM ACC (B). The seedlings were grown in liquid MS medium as described in Materials and methods. *UBQ5* was amplified as a control for the amount of template. Cycle numbers refer to number of PCR reaction to amplify each cDNA.

In Arabidopsis, glc represses expression of many genes involved in ethylene biosynthesis and signaling (Price et al. 2004). We therefore compared the transcript levels of *ACCOX*, *ACCS* and *PEROX* genes in seedlings of HKL1 transgenic lines and mutant with or without glc and ACC treatments. *ACCOX* and *ACCS* encode enzymes in

the biosynthesis of ethylene and these specific family members are reported to be repressed by glc (Price et al. 2004). *PEROX* encodes a putative peroxidase involved in oxidative stress responses and is induced by ACC treatment (Nemhauser et al. 2005), but is not an apparent target of glc signaling. In our hands, glc treatment of Col seedlings also did not affect the transcript of *PEROX*, but it did repress the *ACCS* mRNA strongly and the *ACCOX* mRNA weakly. In *hkl1-1* seedlings also, the expression of *PEROX* was not affected by glc treatment. However, in contrast to WT Col seedlings, the transcripts of *ACCS* and A*CCOX* were not repressed by glc in *hkl1-1* seedlings (Fig 4.4 A).

ACC treatment induced the transcript of *PEROX* but did not alter the expression levels of *ACCOX* and *ACCS* (Fig. 4.4 B), in both Col and *hkl1-1* seedlings. The RT-PCR results of Ler, HKL1-HA, gin2-1, and HKL1-FLAG from both glc and ACC treatment were not consistent in all the replicates, hence are not shown here. However, the results of *hkl-1* indicate that HKL1 is somehow required for the glc dependent repression of *ACCS* and *ACCOX*; however, it is not required for the induction of *PEROX* by ethylene.

HKL1 overexpression lines and gin2-1 have a glucose dependent root hair phenotype

In plants, seedling root hairs are an excellent system to study cell growth and development as they are easily accessible, single celled, and can be microscopically analyzed without disturbing the surrounding tissue. In addition to the growth phenotypes of the HKL1 transgenic lines and mutants reported earlier (Chapter 3), we also observed a conditional root hair phenotype in seedlings of HKL1 overexpression lines and in *gin2-1*. In this experiment, 7 d old seedlings grown on 0.5% suc or 2% glc were transferred to

6% glc plates and the root hair morphology was examined thereafter. Col and Ler seedlings grown on 0.5% suc or 2% glc plates produced slender and tubular root hairs without any observable defects. Seedling transfer from 2% to 6% glc or 0.5% suc to 6%

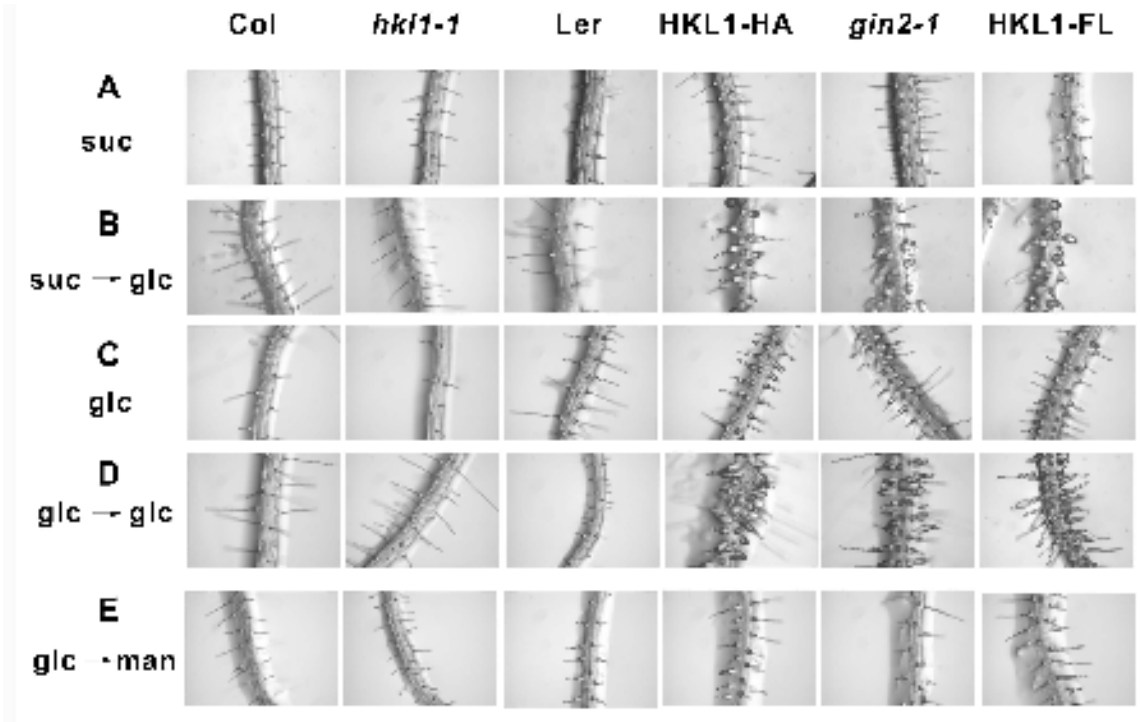

Figure 4.5 Root hair phenotypes of HKL1 overexpression lines and *gin2-1* on different sugar plates. The root hairs of Col, *hkl1-1*, Ler, HKL1-HA, *gin2-1* and HKL1-FLAG seedlings grown under differ growth conditions. (A) Seedling grown on plates with 0.5% suc plates. (B) Seedling transferred from plates with 0.5% suc to 6% glc plates. (C) Seedlings grown on 2% glc plates. (D) Seedlings transferred from plates with 2% glc to plates with 6% glc. (E) Seedlings transferred from 2% glc plates to plates with 6% mannitol. Seedlings were grown vertically under constant light (30 $\mu mol\, m^{-2}s^{-1}$). At least 10 seedlings of each genotype grown for 7 d on 0.5% suc or 2% glc plates were used for the transfer in three separate experiments.

glc also did not alter the root hair morphology of WT seedlings (Fig. 4.5 B, D).

Seedlings of *hkl1-1* produced root hairs which were morphologically similar to WT Col

on 0.5% suc and 2% glc, or upon transfer from either condition to 6% glc. Seedling root hairs of HKL1-HA, *gin2-1* and HKL1-FLAG lines grown on 0.5% suc plates also were normal (Fig 4.5A). However, when these lines were grown on 2% glc, the root hairs were often thicker than normal, with a small bulge at the base of (Fig 4.5 C). Even more strikingly, HKL1-HA, *gin2-1* and HKL1-FLAG seedlings transferred from 2% to 6% glc produced abnormally thick and bulbous root hairs, which were not observed in transferred WT or *hkl1-1* seedlings (Fig 4.5 B, D). The HKL1-HA, *gin2-1* and HKL1-FLAG seedlings transferred from the 0.5% suc plates to 6% glc, also produced bulbous and thick root hairs. However, seedlings transferred from suc plates showed a 1-2 d delay in developing this phenotype. In contrast, seedlings of all the genotypes transferred from 0.5% suc or 2% glc to 6% mannitol did not produce root hairs with any growth abnormalities (Fig 4.5 E). This indicates that bulbous root hairs are not due to the osmotic stress, but are in response to increased levels of glc.

Mutations in the vegetative *ACTIN2* (*ACT2*) cause a hairless or short root hair phenotype, with a bulge at the base of the root hairs (Gilliland et al. 2002; Nishimura et al. 2003). Since glc has been shown to modulate the actin cytoskeleton (Balasubramainan et al. 2007), we therefore screened *act2-1* seedlings under the conditions that produced bulbous root hairs in HKL1 overexpression lines and in *gin2-1*. The *act2-1* seedlings had fewer root hairs than WT Ws seedlings on 0.5% suc, 2% glc, or 6% glc (Fig. 4.6). However, *act2-1* seedlings did not show the bulbous root hair phenotype upon transfer from 0.5% suc to 6% glc (data not shown) or from 2% glc to 6% glc (Fig. 4.6), as otherwise observed in the HKL1 overexpression lines and in *gin2-1*

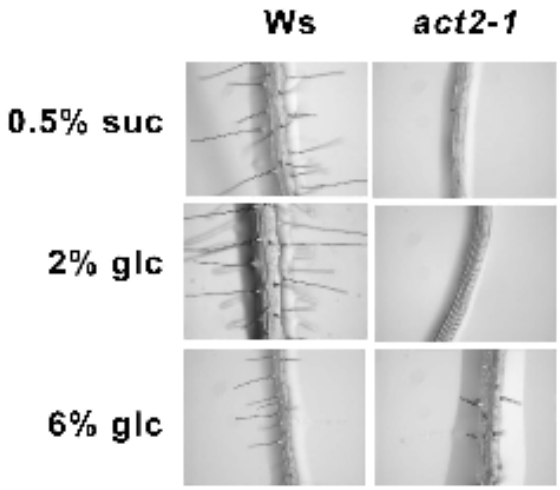

Figure 4.6 Root hair morphology of *act2-1* seedlings grown on different sugar plates. Seedlings of WT Ws and *act2-1* were grown on different sugar plates or transferred from 2% glc plates to 6% glc plates. At least 10 seedlings of each genotype grown for 7 d on were used for the transfer in three separate experiments.

(Fig. 4.5). These results suggest that the phenotypic response in HKL1 overexpression lines and *gin2-1* might not be directly due to disruption of the actin cytoskeleton.

Ethylene has a role in the bulbous root hair phenotype of HKL1-HA

In Arabidopsis root hair development and growth is controlled by multiple plant hormones, including ethylene (Zhu et al. 2005). The phenotypes and the transcript data suggested that HKL1 might mediate cross-talk between ethylene and glc signaling. Therefore, we tested whether ethylene also has role in the glc-dependent root hair phenotype of HKL1 overexpression lines and *gin2-1* seedlings. Seedlings of HKL1 transgenic lines and mutants were grown on 0.5% suc or 2% glc for 7 d and then transferred to MS plates containing 0.5% suc + 5 μM ACC and to plates with 2% glc + 5

μM ACC, respectively. The Col and Ler seedlings grown on 0.5% suc or 2% glc with ACC produced significantly more root hairs (Fig. 4.7 A, B) compared to control plates without ACC (Fig. 4.5 A, C). This proliferation response was previously observed by Tanimoto et al. (1995) for Col seedlings on suc plates with ACC. The *hkl1-1*seedlings were less responsive to ACC treatment and produced fewer root hairs on ACC plates

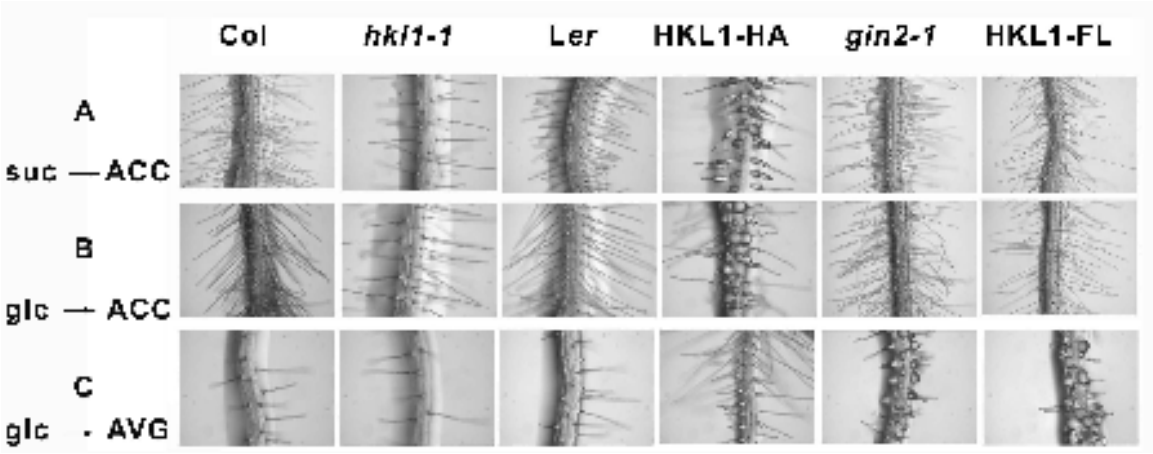

Figure 4.7 Role of ethylene in the root hair phenotype of HKL1-HA. (A) The root hairs of the Col, *hkl1-1*, Ler, HKL1-HA, *gin2-1* and HKL1-FLAG seedlings after transferred from 0.5% plates suc plates to 0.5% suc plates supplemented with 5μM ACC. (B) Root hairs of seedlings in A transferred from 2% glc plates to 2% glc plates with 5μM ACC. (C) Root hairs of seedling transferred from 2% glc plates to 6% glc plates with 1μM AVG. At least 10 seedlings of each genotype were used for the transfer in three separate experiments.

compared to WT Col seedlings. This is consistent with the observed insensitivity of *hkl1-1* to ACC previously observed on glc plates (Fig. 4.2). Interestingly, seedlings of HKL1-HA, produced bulbous root hairs upon transfer to 0.5% suc and 2% glc plates with ACC (Fig 4.7 A, B), as observed in the HKL1-HA seedlings transferred from 0.5% suc or 2% glc to 6% glc plates (Fig. 4.5 B, D). However, seedlings of *gin2-1* and HKL1-FLAG did not produce any bulbous root hairs upon transfer to ACC plates (Fig. 4.7A, B).

These results indicate that ethylene can produce a similar phenotypic response as glc in HKL1 overexpression lines, but only if HXK1 is present.

Since ethylene is involved in the root hair phenotype of HKL1-HA seedlings grown on plates with high glc, we tested whether blocking ethylene biosynthesis might rescue the phenotype. For this experiment, the seedlings grown on 2% glc plates were transferred to 6% glc plates with the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG). The AVG treatment did not alter the root hair morphology of Col, Ler and *hkl1-1* seedling (Fig. 4.7 C, D). The seedlings on these plates produced slender and tubular root hair similar to the ones observed on plates without AVG (Fig. 4.5 C). Interestingly, in HKL1-HA seedlings transferred to 6% glc plates with AVG the bulbous root hair phenotype was greatly attenuated. Instead of forming bulbous root hairs, these seedlings produced slender root hairs similar to WT Ler seedlings on 6% glc (Fig. 4.7 D). In contrast, seedlings of *gin2-1* and HKL1-FLAG transferred to AVG plates produced similar bulbous root hairs as observed on control plates with 6% glc (Fig. 4.5 B, D). The response of HKL1-HA to AVG indicates that the bulbous root hair phenotype of HKL1-HA might be due to increased seedling ethylene production.

Ethylene overproduction mutant eto2-1 shows altered root hair morphology

To further test the involvement of ethylene in the root hair phenotype, we examined root hairs of two ethylene mutants, a strong ethylene signaling mutant *ein2-1*

(Alonso et al. 1999) and an ethylene overproduction mutant *eto 2-1* (Vogel et al. 1998). Seedlings of *ein2-1* when grown on plates with 0.5% suc and 2% glc (Fig. 4.8 A) produced hardly any root hairs. Seedlings of *eto2-1* on the other hand produced a significantly greater number of root hairs compared to WT Col seedling on 0.5% suc or 2% glc, but similar to WT Col grown on plates with ACC (Fig. 4.8 B, compare with Fig. 4.5 A, C and Fig 4.7 A). This is consistent with the enhanced root hair production by WT seedlings treated with ACC (Fig. 4.7 A). However, the root hair morphology was not affected in either *ein2-1* or *eto2-1* seedlings grown continuously on 0.5% suc or 2% glc plates (Fig 4.8 A, B). When seedlings grown on 2% glc were transferred to 6% glc plates, the *ein2-1* seedlings produced root hairs with normal root hair morphology. Interestingly, *eto2-1* seedlings transferred to 6% glc plates produced some bulbous root hairs (Fig 4.8 B). This root hair phenotype of *eto2-1* was somewhat different from that of the HKL1-HA seedlings transferred to 6% glc plates. In HKL1-HA root hairs, the bulge was localized more towards the base of the root hair (Fig. 4.5 D), while in *eto2-1*, the bulge was more towards the tip of the root hairs. To further study the role of glc in the root hair phenotype of *eto2-1* we observed the root hairs of the *eto2-1* and WT Col seedlings transferred to MS plates containing even higher concentrations of glc after initial growth on 2% glc plates. In the presence of 7% glc, *eto2-1* seedlings produced short and bulbous root hairs not observed in Col (Fig. 4.8 C). Further increase in glc concentration to 8% did not alter the root hair morphology of Col seedlings. However, *eto2-1* seedlings transferred to 8% glc produced short and bulbous root hairs similar to the ones observed in the seedlings of HKL1 overexpression lines or *gin2-1* (Fig. 4.8 C).

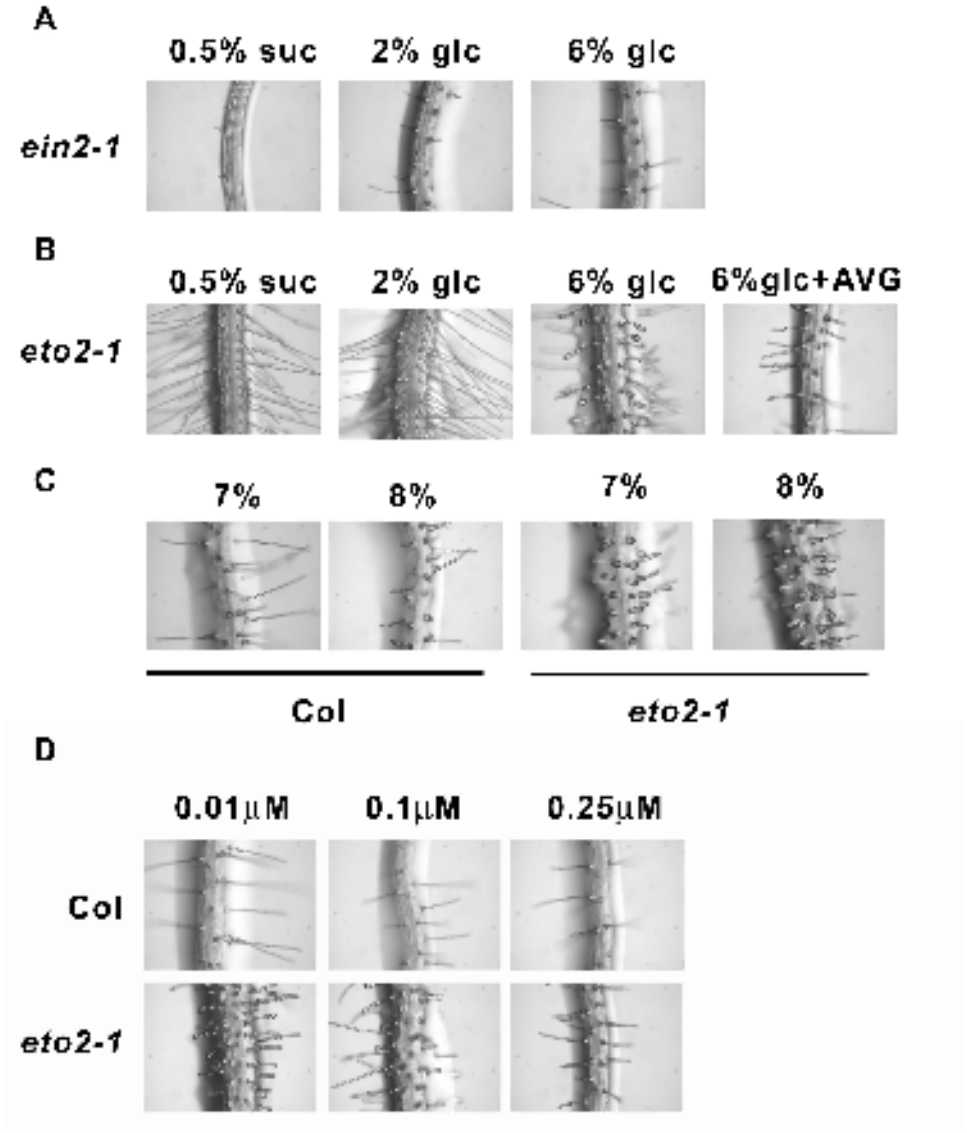

Figure 4.8 Root hair growth of *ein2-1* and *eto2-1*. (A) Seedlings of *ein2-1*grown continuously on 0.5% suc plates, 2% glc plates and transferred from 2% glc to 6% glc plates. (B) Seedling of *eto2-1* grown continuously on 0.5% suc plates, 2% glc plates, and seedlings transferred from 2% glc to 6% glc with or without 1μM AVG (C) Seedlings of Col and *eto2-1* transferred from 2% glc plates to 7% and 8% glc plates (D) Seedlings of Col and *eto2-1* transferred from 2% glc plates to 6% glc plates supplemented with 0.01μM, 0.1 μM, 0.25 μM AVG. At least 10 seedlings of each genotype were used for the transfer in three separate experiments.

These results indicate that the bulbous root hairs observed in *eto2-1* are also regulated at least in part by both glc and ethylene.

Since *eto2-1* seedlings produce 10- 40 fold more ethylene than WT seedlings (Chae et al. 2003) we further examined whether blocking ethylene biosynthesis would rescue the bulbous root hair phenotype of *eto2-1* seedlings. The *eto2-1* seedlings transferred to 6% glc plates with 1 μM AVG produced fewer roots than seedling on 6% glc alone. Furthermore, seedlings on AVG produced normal root hairs without the bulge at the root hair tips (Fig. 4.8 B), indicating that ethylene does have a role in forming bulbous root hairs observed in *eto2-1*. Next, we studied the response of varying AVG concentration on the bulbous root hair phenotype. The WT Col seedlings produced fewer root hairs on 6% glc plates with 0.001, 0.1 and 0.25 μM AVG, but their root hair morphology was not altered due to AVG treatment (Fig 4.8 D). In *eto2-1,* bulbous root hairs were produced in the seedlings transferred to plates with 0.001 and 0.1 μM AVG but not on the plates with 0.25 μM AVG (Fig. 4.8 D). These results of AVG treatment indicate that the bulbous root hairs observed in *eto2-1* on glc plates are associated with enhanced ethylene biosynthesis.

Taken together these root phenotypes reveal a novel interaction between glc and ethylene in regulating root hair growth. Based on the described root hair phenotypes we surmise that such an interaction is functionally relevant.

HKL1 interacts with HXK1 in co-immunoprecipitation assay.

In contrast to responses of the HKL1-HA line, the bulbous root hair phenotype could not be induced by ethylene or suppressed by AVG in the HKL1-FLAG line which lacks HXK1. The growth phenotypes of HKL1 transgenic lines and mutant indicate that HKL1 acts as a negative regulator of plant growth when HXK1is present (Chapter3; Fig. 3.2-3.6). Both HXK1 and HKL1 are localized on mitochondria (Heazelwood et al. 2004; Karve et al. 2008). Therefore, we tested whether HKL1 and HXK1 can interact with each other and therefore might form a functional complex. To do this, we carried out co-immunoprecipitation assays after transiently expressing HXK1-HA plus or minus transfected HKL1-GFP in maize mesophyll protoplasts. Since HXK1 and HKL1 are both ~50 kD in size, addition of the GFP tag helped in size separation of the proteins on the SDS gels. In control assays, anti-HA antibody did not capture HKL1-GFP protein and anti-GFP antibody did not capture HXK1-HA protein (Fig. 4.9). However, anti-HA antibody did pull down HXK1-GFP from protoplasts that transiently expressed both HXK1-HA and HKL1-GFP. Similarly, anti-GFP antibody could pull down HXK1-HA from the protoplasts that expressed both HXK1-HA and HKL1-GFP (Fig. 4.9). As a possible control, we did comparable assays between co-transfected YHK2-HA, and HKL1-GFP. The anti-HA antibody did precipitate YHK2-HA, but when both the proteins were expressed did not pull down HKL1-GFP (Fig. 4.9). These results indicate that Arabidopsis HKL1 and HXK1 can interact. Based on the described phenotypes of the HKL1 transgenic lines and mutants, we surmise that such an interaction is functionally relevant.

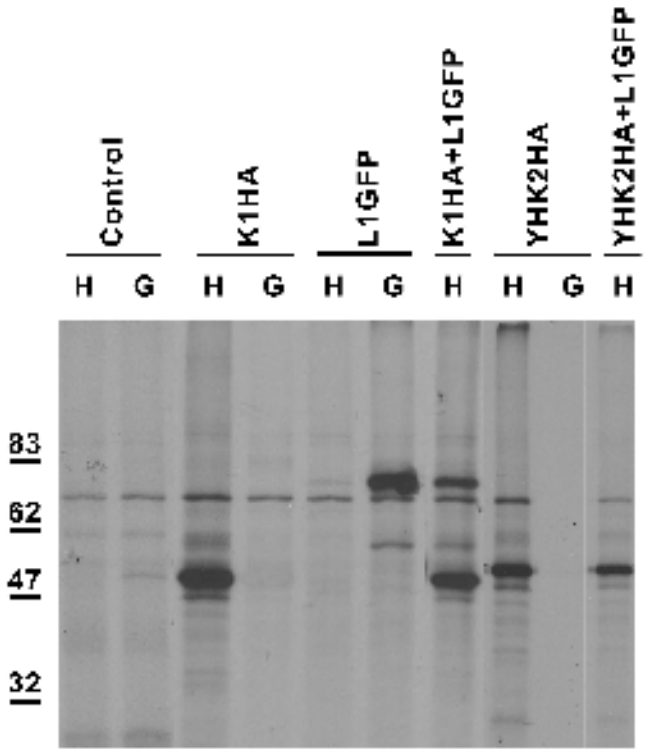

Figure 4.9 Co-immunoprecipitation assay for interaction between HKL1 and HXK1 after their transient expression in maize mesophyll protoplasts. Protoplasts were transfected with HKL1-GFP (L1 GFP) and/or HXK1-HA (K1 HA), and/ or YHK2-HA (YHK2HA) and labeled with [^{35}S]Met. Pulldown assays were done using anti-HA antibody (H) or anti-GFP antibody (G). Control, untransfected protoplasts.

Discussion

Previous characterization of HKL1 transgenic and mutant lines has shown that even though HKL1 is a non catalytic protein, its expression influences plant growth (Chapter 3). In HKL1, the glc binding domain and other structural features required for binding to glc are largely conserved. Some of the HKL1 associated phenotypes are glc

dependent but these were suggested not to be due to altered glc signaling. These observations were extended in the present study by demonstrating that the repression response of known glc response genes, *ASN1, GLYK* and *T6PSY* was not affected in transgenic and mutant lines with altered HKL1 protein expression. Among these 3 genes, *ASN1* was repressed in *gin2-1* whereas other two were not repressed. Therefore we conclude that HKL1 likely does not affect glc-dependent regulation of genes by either HXK1-dependent or independent signaling processes.

These results however do not explain the altered glc sensitivity of HKL1 transgenic and mutant lines. As one possible factor, we examined the role of ethylene in some of the glc responses of the HKL1 transgenic and mutant lines. Interestingly, the *hkl1-1* mutant was found to be relatively ethylene insensitive while the HKL1-HA line was relatively hypersensitive to ethylene. For example, in WT Arabidopsis ACC treatment can overcome the seedling developmental arrest induced by glc (Fig 4.2; Zhou et al. 1998). Seedlings of *hkl1-1*, however, were insensitive to the ACC. On plates with 6% glc + ACC seedlings of HKL1-HA had reduced root length, cotyledon growth and increased root swelling.

In Arabidopsis, the glc response phenotypes of other transgenic and mutants have often been linked to cross-talk with other hormone signaling pathways (Gibson, 2004). Analysis of the glc insensitive mutant *gin1* originally revealed that cross-talk occurs between glc and ethylene signaling (Zhou et al. 1998). GIN1 was later found to be allelic to *aba2*, which encodes a short-chain alcohol dehydrogenase involved in ABA biosynthesis (Cheng et al. 2002). Interestingly, *hkl1-1* is relatively hypersensitive to

ABA inhibition of seed germination (Appendix D). It would be interesting to know whether HKL1 transgenic lines also have altered responses to ABA.

To test whether HKL1 can influence expression of known ethylene response or biosynthesis genes, we measured the transcript levels of *SP2* and *LRR* genes in seedlings of HKL1 transgenic and mutant lines after ACC and glc treatments. The transcripts of *SP2* and *LRR* have been shown to be induced and repressed, respectively, by ethylene treatment (Nemhauser et al. 2005). However, in our experiment both transcripts were repressed by ACC treatment (Fig. 4.3). This might be due to the small differences in the experimental design and treatments. We treated seedlings for a relatively longer time (8 h vs 30 min) and with a relatively higher ACC concentration (50 μM vs 10 μM), similar to what was used in the glc repression assays. Importantly, *SP2* and *LRR* transcripts were not repressed in *hkl1-1*seedlings by ACC treatment. We also found that *SP2* transcript was glc repressed in WT seedlings, but was not repressed in *hkl1-1* seedlings. Furthermore, that glc dependent repression of two ethylene biosynthesis genes (ACC synthetase and ACC oxidase) also was not affected in *hkl1-1*. Therefore, we conclude that HKL1 is required for ethylene regulation of certain target genes and is required for glc regulation of at least some genes associated with ethylene metabolism.

In this study we also describe a novel root hair phenotype not previously seen with other transgenic or mutant lines associated with glc or ethylene responses. In the described assay, *gin2-1* and both of the HKL1 overexpression lines produced root hairs with a bulbous swelling at the base when grown on plates with 6% glc (Fig. 4.5). Furthermore, the root hair phenotype of HKL1-HA could be phenocopied by ACC and

blocked by AVG (Fig. 4.7). Ethylene acts as a positive regulator of root hair formation (Tanimoto et al. 1995). The *hkl1-1* showed a relative insensitivity to ACC induced root formation in the root hair response assay. Since HKL1-HA also produced shorter roots and cotyledons compared to *gin2-1* or HKL1-FL on 6% glc plates with ACC, the root hair phenotype of HKL1-HA might be due to the observed ethylene hypersensitivity. Ethylene has been shown to modulate the growth of root hairs and epidermal cells (Zhu et al. 2006). The bulbous root hairs were also seen in *gin2-1* or HKL1 overexpression in *gin2-1* on glc plates (Fig. 4.5). However, in this case the phenotype could not be rescued by blocking ethylene biosynthesis. Therefore, this response, like several other phenotypes of HKL1, is also dependent on HXK1 protein.

Using known ethylene biosynthesis and response mutants, we have established that this novel root hair response depends as well on ethylene biosynthesis. The absence of the bulbous root hairs in seedlings of ethylene overproducing line *eto2-1* grown on suc plates and the observed glc-dependent formation of bulbous root hairs (Fig. 4.8) indicate that glc and ethylene are both involved this response. Since AVG blocked the bulbous root hair phenotype in both HKL1-HA and *eto2-1*, it is possible that glc acts in this process by modulating ethylene biosynthesis. Transcripts of several genes involved in ethylene biosynthesis and signaling have been shown to be regulated by glc treatment (Price et al. 2004). The application of exogenous ethylene has been shown to suppress aberrant root hair development in *rhd1* (Seifert et al. 2004) and *der8* (Ringli et al. 2005). Both of these mutants produce bulbous root hair on suc plates in the absence of ethylene,

but produced normal root hairs on plates with ACC. Our results show that ethylene in combination with glc can stimulate the formation of aberrant root hairs.

Root hair growth is a dynamic process and requires a well organized network of actin filaments and microtubules. In general, actin filaments are involved in root hair expansion and microtubules are required for the directional orientation of the root hair (Sieberer et al. 2005). One possibility is therefore that the observed bulbous root hairs are associated with the cytoskeleton of the root hair. However, in this study we did not observe the bulbous root hair phenotype in *act2-1*. Interestingly, glc disrupts organization of actin filaments (Balasubramanian et al. 2007) and it might lead to the rearrangements of the actin cytoskeleton in root hairs in HKL1-HA, HKL1-FL and *gin2-1* seedlings. Therefore the observed bulbous root hair phenotype can possibly affected by the HXK1 and HKL1 through interaction with F-actin in a glc dependent manner.

In addition to the role of plant hormones, reactive oxygen species (ROS) also play a critical role in root hair growth (Carol and Dolan, 2006). Tip localization of ROS and ROP2, a member of Rho GTPase, is required for the normal root hair growth (Carol et al 2005). Furthermore, ROP2 is involved in polarized growth of root hairs by coordinating actin and microtubule organization (Yang et al. 2007). In Arabidopsis, regulation of cell elongation by ethylene is dependent on ROS accumulation (De Cnodder et al. 2005). Recently, it has been the shown that glucoseamine induced accumulation of ROS is repressed in *gin2-1* (Ju et al. 2008). Interestingly we observed reduced expression of *PEROX* which encodes a putative peroxidase involved in generating of ROS in *gin2-1*, and *PEROX* was repressed by glc treatment in both the HKL1 overexpression lines (data

not shown). It is therefore possible that glc and ethylene treatment somehow regulate the generation of ROS in the root hair which in turn regulate the root hair growth phenotype.

Most of the observed growth phenotypes of HKL1 reported previously (Chapter 3) were dependent on the presence of HXK1 protein. In this regard, we have shown that by co-immunoprecipitation assay, HKL1 can specifically interact with HXK1. HXK isoforms have been shown to form dimers and oligomers in humans, yeast and recently in Trypanosomes. In *Trypnasoma bruci*, catalytically inactive HXK2 exhibits activity in a complex with catalytically active HXK1 (Chambers et al. 2008). We did not see any change of activity by expressing HKL1 and HXK1 cDNA in different ratios in leaf protoplasts (Karve and Moore, unpublished data). However the growth phenotypes of HKL1 transgenic lines support the idea that the interaction between HKL1 and HXK1 has a regulatory significance and might be essential for the function of HKL1. So far, we have shown by various phenotypic assays and gene expression analysis that HKL1 regulates plant growth at least in part by interaction with ethylene signaling, and it possibly mediates glc responses to some of the ethylene biosynthesis and signaling genes. However, we still do not know whether HKL1 can bind to glc or to any other hexose sugars. Further biochemical analysis of will therefore help in establishing the regulatory role of this non catalytic HXK from Arabidopsis.

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SUMMARY

Hexokinase (HXK) catalyzes the first step in glucose utilization, the conversion of glc to glc 6-P. In addition to its role in catalysis, HXK acts as a glc sensor and regulates plant growth and development. The *Arabidopsis thaliana* genome encodes six HXK genes, of which HXK1 and HXK2 have been shown previously to function as catalytically active proteins and also as glc sensors. The other four HXK gene family members are ~50% identical at the amino acid level with HXK1. When expressed in maize protoplasts, one of the four (HXK3) was found to be catalytically active for hexose phosphorylation. However, the other three were designated as hexokinase-like (HKL) proteins since they lack glucokinase activity (HKL1, HKL2 and HKL3). In this study we examined the characteristics and function of HKL1.

An analysis of the amino acid sequences showed a broad pattern of conserved key motifs and residues among HXK1, HXK2, and HXK3 predicted proteins. In contrast, HKL1 and HKL2 proteins had most of the residues known to be important for sugar binding, but they did have a number of residue changes throughout the protein and also had an indel at the adenosine binding domain. HKL3 protein, on the other hand, lacked many recognized residues important for sugar and adenylate binding and for enzyme catalysis. To explore how these differences between HXK1 and HKL1 might affect catalysis, we employed site-directed mutagenesis to change key amino acids in HXK1 relative to corresponding residues in HKL1. The glucokinase activity of HXK1 was largely compromised by most of the amino acid changes. However, based on the primary

amino acid sequence, HKL1 was predicted to bind glc and to have the flexibility required to undergo a glc dependent conformational change, as observed in HXKs with catalytic activity. These results indicate that HKL1 might be involved in some alternate glc dependent functions such as glc signaling.

To understand the function of HKL1 in organismal space, a reverse genetics approach was used. Transgenic Arabidopsis plants that overexpress epitope tagged HKL1 were generated in WT Ler background (HKL1-HA) as well as in HXK1 null mutant, *gin2-1* background (HKL1-FLAG). We also identified a loss of function T-DNA knockout line in WT Col background (*hkl1-1*). Modulating HKL1 expression severely affected plant growth. For example, HKL1 overexpression in Ler resulted in a severe reduction in rosette size under a long photoperiod and decreased growth of seedling hypocotyls. Conversely, the *hkl1-1* line had reduced rosette size under a short photoperiod and longer seedling hypocotyls. The phenotypes of HKL1-HA were mostly similar to those reported for *gin2-1*. HKL1 overexpression in *gin2-1*, however, did not generally affect the phenotypic responses of the parental line. Based on these results, we suggest that HKL1 is a negative regulator of plant growth, but that its effect largely depends on the presence of HXK1. These growth phenotypes indicated that HKL1 possibly influences other hormone signaling pathways. HKL1 promoter expression analysis indicated further possible interconnections between hormones and HKL1 functions. In particular, HKL1 promoter activity was induced by treatment with ethylene precursor, ACC, and by cytokinin, but it was repressed by ABA.

A possible regulatory role of HKL1 in glc signaling was tested first by growing seedlings on agar plates with 6% glc. In contrast to growth responses of their parental background lines, seedlings of the HKL1-HA line were glc tolerant, whereas those of *hkl1-1* were hypersensitive to glc induced developmental arrest. Seedlings of HKL1-FLAG were also glc tolerant, as were parental *gin2-1* seedlings. These responses indicated that HKL1 might be a negative regulator of glc signaling, depending on the presence of HXK1. We then tested whether the promoter activity of *pRBCS*-LUC, which is a known target of HXK1-dependent glc signaling, is affected by altering HKL1 expression in Arabidopsis protoplasts. However, changing HKL1 expression did not affect signaling to *pRBCS*. As an alternative approach, semi quantitative RT-PCR was used to measure glucose regulated transcripts of *ASN, GLYK* and *T6PSY* from seedlings grown in liquid culture. However, glc repression of these genes was not affected in seedlings of the HKL1 transgenic lines and mutant. Therefore, while HKL1 acts genetically downstream of HXK1 to block seedling developmental arrest, HKL1 has no direct function in glc signaling.

There is much evidence which indicates that glc signaling can affect plant growth by modulating other hormone signaling pathways. For example, an antagonistic interaction between ethylene and glc signaling has been shown previously based on the observation that WT Arabidopsis seedlings can overcome the seedling developmental arrest induced by 6% glc when grown in the presence of ACC. To test whether HKL1 has a role in glc and ethylene cross-talk, seedlings of HKL1 transgenic lines and mutant were grown on 6% glc plates with ACC. Interestingly, ACC treatment could not

overcome the developmental arrest response of *hkl1-1*seedlings. In contrast, seedlings of HKL1-HA, HKL1-FLAG and *gin2-1* showed signs of ethylene hypersensitivity based on reduced primary root growth and radial swelling of the hypocotyl. These results indicated that HKL1 mediates cross-talk between ethylene and glc signaling during seedling development and might modulate some aspects of seedling ethylene sensitivity.

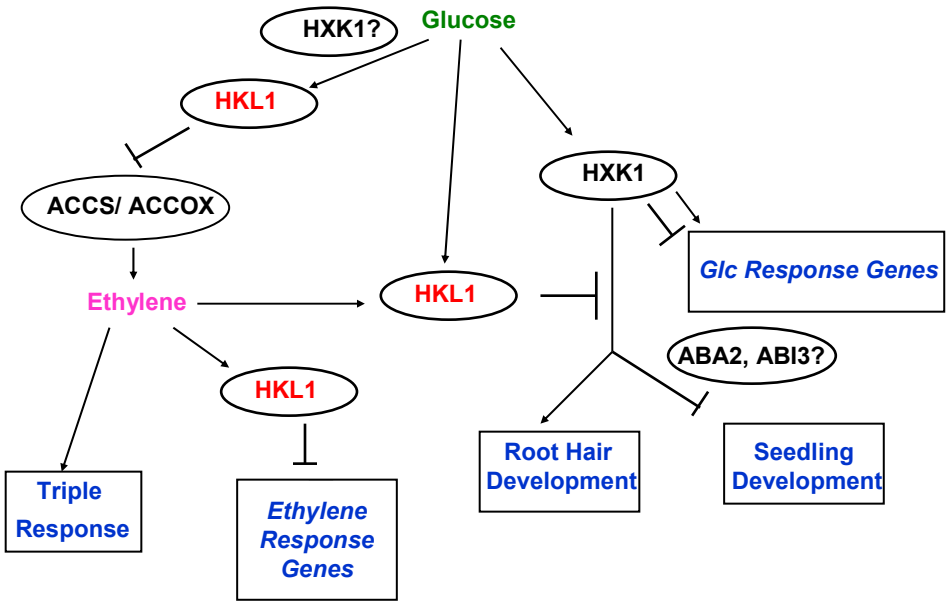

Figure 5.1 A proposed model for HKL1 function. HKL1 acts as a negative regulator of glc dependent developmental processes such as seedling and root hair development downstream of HXK1. Since both seedling development and root hair development are affected in HKL1 transgenic lines and mutant by blocking ethylene biosynthesis, HKL1 facilitates cross-talk between glc and ethylene signaling pathways. HKL1 is required for regulation of some of the ethylene response genes as well for glc repression of ethylene biosynthesis genes *ACCS* and *ACCOX* . Because precise convergence points for ethylene signaling and glc signaling are currently unknown, HKL1 has been shown to act possibly at multiple locations in the model. ABA2- ABA synthase 2; ABI3- ABA insensitive 3; ACCOX- ACC oxidase; ACCS- ACC synthase, HXK1- hexokinase 1; HKL1- hexokinase-like 1.

In Arabidopsis, glc has been shown to regulate the expression of genes involved in ethylene biosynthesis and signaling. However, glc dependent repression of ethylene biosynthesis genes *ACCS* and *ACCOX* was not observed in *hkl1-1* seedlings. These results indicate that HKL1 is somehow required for the glc repression of these genes. Based on the observed dependence on HXK1 for many of the HKL1 growth phenotypes, we speculate that HXK1 is also involved in this process, though that remains to be determined.

In addition to the seedling growth arrest phenotype, a role of HKL1 in mediating cross-talk between glc and ethylene signaling was revealed by a bulbous root hair phenotype. HKL1 overexpression lines and *gin2-1* produced bulbous root hairs when transferred to plates with 6% glc after initial growth on plates with sucrose or 2% glc. Interestingly, transfer to plates with ACC also resulted in bulbous root hairs in the seedlings of HKL1-HA, but not in *gin2-1* or HKL1-FLAG. These results indicate that HXK1 is a positive regulator of root hair development on glc plates, and that HKL1 acts as a downstream negative regulator. Similar bulbous root hairs were also observed in the ethylene overproducing mutant *eto2-1* when grown on glc plates. These results indicate that root hair development in Arabidopsis is regulated by a complex interaction between glc and ethylene, and that HKL1 likely is a component of this signaling cross-talk.

Based on the available experimental evidence, we propose a model to describe the functions of HKL1 (Fig. 5.1). We have shown that HKL1 acts as a negative regulator of glc dependent seedling arrest and root hair development, and that HKL1 functions downstream of HXK1. However, HKL1 likely is not a component of the HXK1-

dependent pathway that is involved in the regulation of glc response genes. Based on the responses of HKL1 transgenic lines and mutant to ACC, HKL1 likely mediates cross-talk between glc and ethylene signaling pathways. This role of HKL1 is also evident by its requirement for glc repression of some ethylene biosynthesis genes. Furthermore, HKL1 was shown to be involved in regulation of some ethylene response genes. However, these genes are likely not regulated by the main ethylene signaling pathway, which regulates the triple response phenotype. The triple response phenotypes are largely unaffected in HKL1 transgenic lines and mutant.

Many of the observed growth phenotypes of HKL1 transgenic lines and mutant were dependent on the presence of HXK1. Both HXK1 and HKL1 showed a similar diverse tissue expression pattern. When expressed in Arabidopsis protoplasts, both HXK1 and HKL1 were localized to mitochondria. We also showed by co-immunoprecipitation assay that HXK1 and HKL1 proteins can physically interact. Although we predict that HKL1 is expressed at a relatively lower level compared to HXK1, the interaction between HXK1 and HKL1 appears to be essential for HKL1 function.

APPENDICES

APPENDIX A

Domains and conserved region of AtHXKs with respect to ScHXK2

	YHK2	HXK1	HXK2	HXK3	HKL1	HKL2	HKL3
Large domain	13-76	27-91	27-91	32-94	27-91	27-91	28-92
	212-457	230-475	230-475	235-476	230-483	231-479	231-470
Small domain	77-211	92-229	92-229	95-234	92-229	92-230	93-230
	458-486	476-496	476-502	477-493	484-498	480-502	471-493
Phosphate 1	82-103	97-118	cons	cons	not cons	not cons	not cons
Loop 1	87-92	102-105	cons	cons	cons	cons	not cons
Loop 2	115-124	not cons	not cons	not cons	not cons	not cons	not cons
Sugar binding core	148-167	167-186	cons	cons	unkn	unkn	not cons
Loop 3	158-163	177-183	cons	cons	cons	cons	not cons
Loop 4	174-178	193-197	cons	cons	cons	cons	not cons
Connect 1	203-223	222-242	cons	cons	not cons	not cons	not cons
Phosphate 2	229-248	248-267	cons	cons	cons	cons	not cons
	Thr234	Thr253	cons	cons	cons	cons	Met
Connect 2	453-473	471-491	cons	cons	cons	cons	cons
Adenosine	411-439	425-461	cons	cons	insert	insert	not cons
	Ser419	Gly441	Gly	Ala	Gly	Gly	Gly
Hydrophobic	Ile85	Leu100	Leu100	Leu103	Leu100	Leu100	Val101
Channel	Leu87	Leu102	Leu102	Leu105	Leu102	Leu102	Leu103
	Leu127	Leu143	Leu143	Leu146	Leu143	Leu143	Leu144
	Ile131	Ile147	Ile147	Ile150	Leu147	Leu147	Ile148
	Leu135	Leu151	Leu151	Leu154	Leu151	Leu151	Leu152
	Ile139	Val155	Val155	Val158	Ile155	Ile155	Leu156
	Leu153	Leu172	Leu172	Leu176	Leu171	Leu172	Leu170
	Phe155	Phe174	Phe174	Phe178	Phe173	Phe174	Phe172
	Phe157	Phe176	Phe176	Phe180	Phe175	Phe176	Phe174
	Phe178	Phe197	Phe197	Phe201	Phe196	Phe197	Asn186
	Leu192	Leu211	Leu211	Leu215	Leu210	Leu211	Met200
	Ile196	Leu215	Met215	Met219	Leu214	Leu215	Leu204
Catalytic residues	Lys176	Lys195	cons	cons	cons	cons	Leu
	Asp211	Asp230	cons	cons	cons	cons	Asn

Continued…

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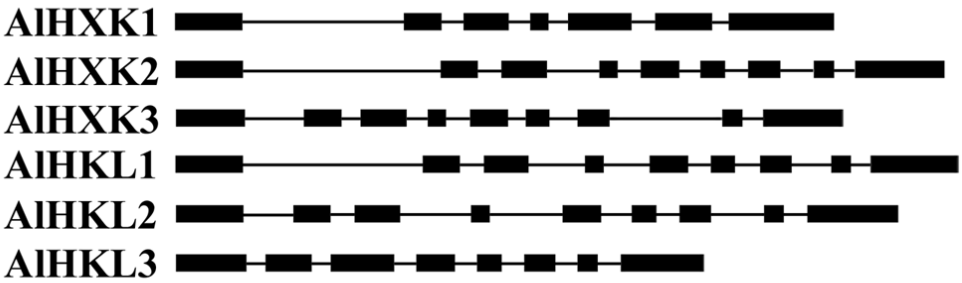
	YHK2	HXK1	HXK2	HXK3	HKL1	HKL2	HKL3
Glucose contacts	Ser158	Ser177	cons	cons	cons	cons	Thr
	Asn237	Asn256	cons	cons	cons	cons	cons
	Glu269	Glu284	cons	cons	cons	cons	cons
	Glu302	Glu315	cons	cons	cons	cons	cons
Conserved Gly	Gly76	Gly91	Gly91	Gly94	Gly91	Gly91	Gly92
	Gly80	Gly95	Gly95	Gly98	Gly95	Gly95	Gly96
	Gly88	Gly103	Gly103	Gly106	Gly103	Gly103	Arg104
	Gly154	Gly173	Gly173	Gly177	Ala172	Ala173	Gly171
	Gly233	Gly252	Gly252	Gly256	Gly251	Gly252	Gly252
	Gly235	Gly254	Gly254	Gly258	Gly253	Gly254	Gly254
	Gly297	Gly310	Gly310	Gly312	Asn309	Asn310	Gly310
	Gly307	Gly320	Gly320	Gly322	Gly319	Gly320	Gly320
	Gly418	Gly440	Gly440	Gly441	Gly447	Gly444	Gly435
	Gly461	Gly479	Gly479	Gly480	Ser486	Ser483	Ala474

Amino acid sequences were aligned using ClustalW (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Annotations are based on regions homologous to yeast hexokinase II (Kuser et al. 2000). Designation of motifs as conserved or not conserved is based on visual inspection and relative assessment of number of amino acid changes. However, the positional information for a given change is not generally known such that some motifs scored as conserved might not be functional. For example, Loop 1 is LGGT. This is changed in HKL2 to LGGS, but scored as conserved. Loop 1 is changed in HKL3 to LRGK and is scored as not conserved. Loop 2 in YHK2 is PDAMRTTQNP. The corresponding region in AtHXK1 is SIPPHLMTGG, but the function might still be retained even though the sequence is completely different. Loop 3 in YHK2 is SFPPASQ, which is present in most of the HXKs as SFPVKQ. This similarity was scored as conserved, except for the much more divergent sequence in HKL3 (TRSVEQ). The conserved core sugar binding motif in YHK2 is LGFTFSF---Q--I, but in AtHXKs the motif is LGFTFSF--Q--L/I. Both HKL1 and HKL2 were scored as having uncertain conservation of the core sugar sequence motif. HKL1 has a sequence of LAFTFSF------I, while HKL2 has a less divergent sequence of LAFTFSF---Q--I. YHK2 = yeast hexokinase 2; HXK1 = At4g29130; HXK2 = At2g19860; HXK3 = At1g47840; HKL1 = At1g50460; HKL2 = At3g20040; HKL3 = At4g37840.

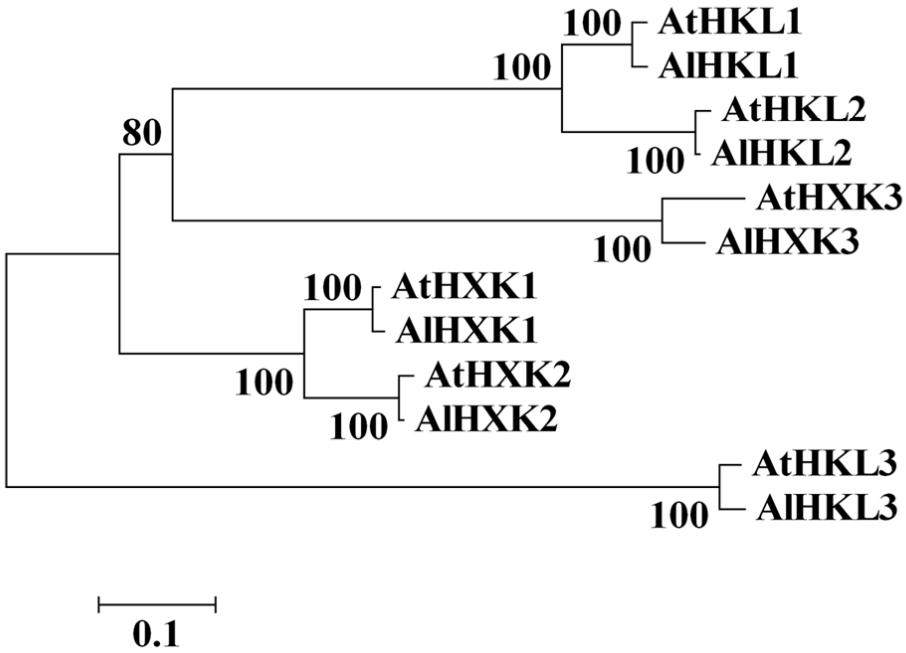
APPENDIX B

Sequence analyses of HXK family genes from *A. lyrata*

A



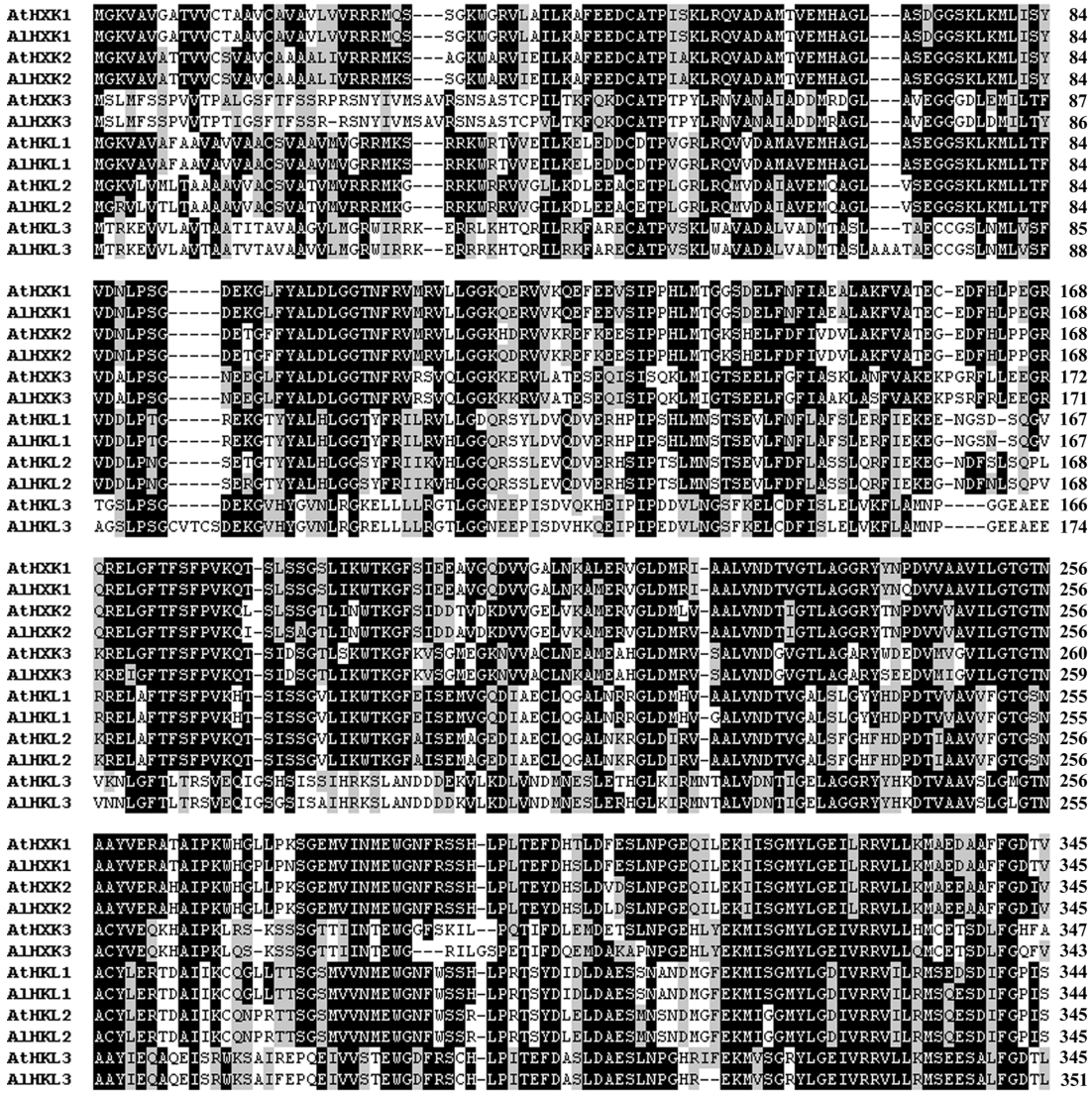
B



(A) Illustrations of gene structures were determined from genomic sequence data. Exons are indicated by rectangles, introns by lines. The lengths of both are proportional to the number of nucleotides that are present. (B) Phylogenetic relationships of predicted HXK family proteins from *A. thaliana* and *A. lyrata*. Phylogenetic reconstruction was done using MEGA4 (Tamura et al. 2007). The *scale bar* = the frequency of amino acid substitutions per unit length.

APPENDIX C

Amino acid sequence alignment for predicted HXK family proteins from *A. thaliana* and *A. lyrata*.



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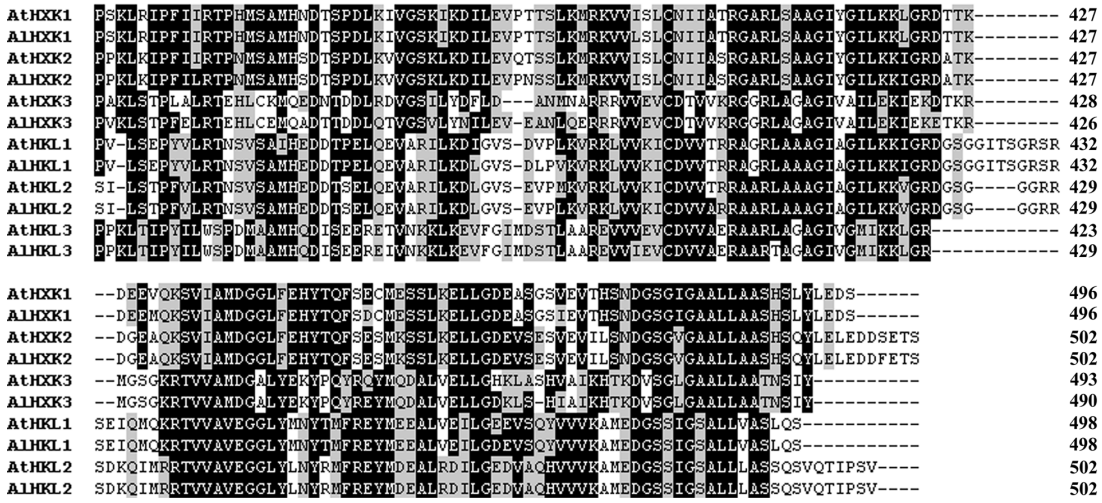
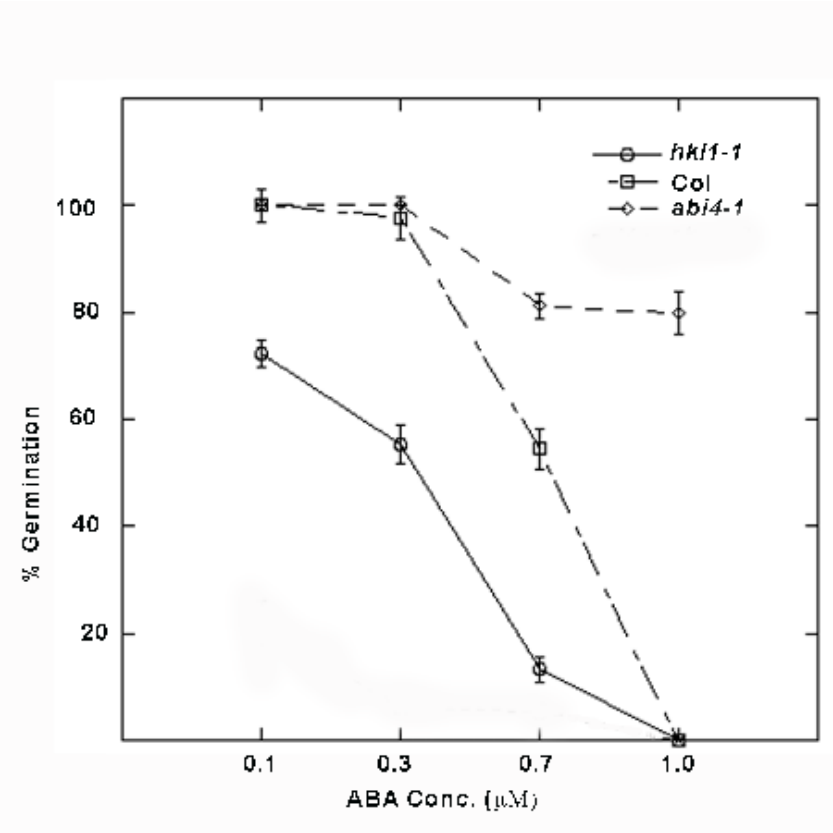

AtHXK1 PSKLRIPFIIRTPHMSAMHNDTSPDLKIVGSKIKDILEVPTTSLKMRKVVISLCNIIATRGARLSAAGIYGILKKLGRDTTK-------- 427
AlHXK1 PSKLRIPFIIRTPHMSAMHNDTSPDLKIVGSKIKDILEVPTTSLKMRKVVLSLCNIIATRGARLSAAGIYGILKKLGRDTTK-------- 427
AtHXK2 PPKLKIPFIIRTPNMSAMHSDTSPDLKVVGSKLKDILEVQTSSLKMRKVVISLCNIIASRGARLSAAGIYGILKKIGRDATK-------- 427
AlHXK2 PPKLKIPFILRTPNMSAMHSDTSPDLKVVGSKLKDILEVPNSSLKMRKVVISLCNIIASRGARLSAAGIYGILKKIGRDATK-------- 427
AtHXK3 PAKLSTPLALRTEHLCKMQEDNTDDLRDVGSILYDFLD---ANMNARRVVEVCDTVVKRGGRLAGAGIVAILEKIEKDTKR-------- 428
AlHXK3 PVKLSTPFELRTEHLCEMQADTTDDLQTVGSVLYNILEV-EANLQERRVVEVCDTVVKRGGRLAGAGIVAILEKIEKETKR-------- 426
AtHKL1 PV-LSEPYVLRTNSVSAIHEDDTPELQEVARILKDIGVS-DVPLKVRKLVVKICDVVTRRAGRLAAAGIAGILKKIGRDGSGGITSGRSR 432
AlHKL1 PV-LSEPYVLRTNSVSAMHEDDTPELQEVARILKDLGVS-DLPVKVRKLVVKICDVVTRRAGRLAAAGIAGILKKIGRDGSGGITSGRSR 432
AtHKL2 SI-LSTPFVLRTNSVSAMHEDDTSELQEVARILKDLGVS-EVPMKVRKLVVKICDVVTRRAARLAAAGIAGILKKVGRDGSG----GGRR 429
AlHKL2 SI-LSTPFVLRTNSVSAMHEDDTSELQEVARILKDLGVS-EVPLKVRKLVVKICDVVARRAARLAAAGIAGILKKVGRDGSG----GGRR 429
AtHKL3 PPKLTIPYILWSPDMAAMHQDISEPRETVNKKLKEVFGIMDSTLAARFVVVEVCDVVAERAARLAGAGIVGMIKKLGR------------ 423
AlHKL3 PPKLTIPYILWSPDMAAMHQDISEPREIVNKKLKEVFGIMDSTLAARFVVIEVCDVVAERAARTAGAGIVGMIKKLGR------------ 429
AtHXK1 --DEEVQKSVIAMDGGLFEHYTQFSECMESSLKELLGDEASGSVEVTHSNDGSGIGAALLAASHSLYLEDS------ 496
AlHXK1 --DEEMQKSVIAMDGGLFEHYTQFSDCMESSLKELLGDEASGSIEVTHSNDGSGIGAALLAASHSLYLEDS------ 496
AtHXK2 --DGEAQKSVIAMDGGLFEHYTQFSESMKSSLKELLGDEVSESVEVILSNDGSGVGAALLAASHSQYLELEDDSETS 502
AlHXK2 --DGEAQKSVIAMDGGLFEHYTQFSESMKSSLKELLGDEVSESVEVILSNDGSGVGAALLAASHSQYLELEDDFETS 502
AtHXK3 --MGSGKRTVVAMDGALYEKYPQYRQYMQDALVELLGHKLASHVAIKHTKDVSGLGAALLAATNSIY---------- 493
AlHXK3 --MGSGKRTVVAMDGALYEKYPQYREYMQDALVELLGDKLS-HIAIKHTKDVSGLGAALLAATNSIY---------- 490
AtHKL1 SEIQMQKRTVVAVEGGLYMNYTMFREYMEEALVELLGEEVSQYVVVKAMEDGSSIGSALLVASLQS---------- 498
AlHKL1 SEIQMQKRTVVAVEGGLYMNYTMFREYMEEALVELLGDEVSQYVVVKAMEDGSSIGSALLVASLQS---------- 498
AtHKL2 SDKQIMRRTVVAVEGGLYLNYRMFREYMDEALRDILGEDVACHVVVKAMEDGSSIGSALLLASSQSVQTIPSV---- 502
AlHKL2 SDKQIMRRTVVAVEGGLYLNYRMFREYMDEALRDILGEDVAQHVVVKAMEDGSSIGSALLLASSQSVQTIPSV---- 502

APPENDIX D

Effect of ABA on seed germination



Seeds of Col, *hkl1-1* and *abi4-1 3* were surface sterilized and stratified for 2d at 4°C. At least 30 seeds of each genotype were germinated on 0.1, 0.3, 0.7, 1.0 μM ABA and germination count was taken 3d after planting. Error bars represent SE.

APPENDIX E

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Requestor Type	Individual
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Expected completion date	Oct 2008
Total	0.00 USD
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