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Regulatory Functions of Nuclear Hexokinase1 Complex in Glucose Signaling

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SUMMARY

Arabidopsis hexokinase1 (HXK1) is a glucose sensor that integrates nutrient and hormone signals to govern gene expression and plant growth in response to environmental cues. How the metabolic enzyme mediates glucose signaling remains a mystery. By coupling proteomic and binary-interaction screens, we discover two nuclear-specific HXK1 unconventional partners: the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B). Remarkably, *vha-B1* and *rpt5b* mutants uniquely share a broad spectrum of glucose response defects with the HXK1 mutant *gin2* (*glucose-insensitive2*). Genetic and chromatin immunoprecipitation analyses suggest that the nuclear HXK1 forms a glucose signaling complex core with VHA-B1 and RPT5B that directly modulates specific target gene transcription independent of glucose metabolism. The findings support a model in which conserved metabolic enzymes and proteins of well-established activities may perform previously unrecognized nuclear functions.

INTRODUCTION

Glucose is one of the most ancient and central signaling molecules in a broad range of organisms from *E. coli* and yeast to humans and plants. The diverse molecular mechanisms of glucose sensing and signaling, as well as glucose-mediated transcription repression and activation, have been intensively studied in unicellular microbes (Stulke and Hillen, 1999; Johnston and Kim, 2005; Moreno et al., 2005; Santangelo, 2006). At least four yeast sensors—Hxk2, glucose transporter-like Snf3 and Rgt2, and G protein-coupled receptor Gpr1—have been identified to transduce internal and external glucose signals to control cell growth and gene expression (Entian, 1980; Lemaire et al., 2004; Johnston and Kim, 2005; Moreno et al., 2005). Despite the essential roles of glucose in gene expression, physiology, metabolism, cell proliferation and death, development, and human diseases, the molecular mechanisms of glucose signal-transduction pathways remain elusive in plants and animals (Rolland et al., 2001, 2006; Danial et al., 2003; Wilson, 2003; Dentin et al., 2004).

The most evolutionarily conserved glucose sensors are HXKs in yeast, mammals, and plants (Moore et al., 2003; Wilson, 2003; Dentin et al., 2004; Moreno et al., 2005). The yeast Hxk2 has been known to play a role in glucose-mediated catabolite repression for more than two decades (Entian, 1980). Recent studies provide new evidence for the nuclear localization of Hxk2 through direct interaction with a DNA binding transcription repressor Mig1, which is regulated through phosphorylation by Snf1 protein kinase and dephosphorylation by Glc7-Reg1 protein phosphatase complex in response to glucose levels. The actions of Hxk2/Mig1/Snf1 appear to be yeast specific, and the precise molecular mechanisms in glucose sensing and signaling remain to be resolved (Ahuatzi et al., 2004; Moreno et al., 2005; Santangelo, 2006). In mammals, novel functions of liver glucokinase (GK/HXKIV) have recently been uncovered, including GK interaction with proapoptotic Bcl-xL/Bcl-2-associated death promoter (BAD) at mitochondria to control apoptosis (Danial et al., 2003) and the requirement of GK in mediating glucose activation of glycolytic and lipogenic genes (Dentin et al., 2004). Although glucose metabolism via GK was suggested to be important for transcription regulation (Dentin et al., 2004), the ability of human GK to complement glucose-signaling defects of the yeast *hxk2* mutant indicates a potential signaling function of mammalian GKs (Mayordomo and Sanz, 2001). Intriguingly, liver and brain GK and HXKIII in many tissues are detected in the nucleus and may serve currently undefined functions (Alvarez et al., 2002; Wilson, 2003).

Sugars play central regulatory roles in many vital processes of photosynthetic plants. Studies of sugar responses in diverse plant species and sugar insensitive or oversensitive mutants in *Arabidopsis* have revealed the

pivotal roles of sugars in the regulation of gene expression; cell proliferation and death; seedling, root, stem, and inflorescence growth; leaf expansion and senescence; and seed development (Koch, 1996; Smeekens, 2000; Gibson, 2005; Rolland et al., 2006). There appears to be multiple pathways for sugar and metabolite or energy sensing and signaling in plants (Smeekens, 2000; Coruzzi and Bush, 2001; Halford et al., 2003; Rolland et al., 2006). Genetic, phenotypic, and biochemical analyses of the *Arabidopsis* HXK1 mutants, *gin2*, have provided compelling evidence for uncoupling glucose-signaling functions from metabolic activities. It has been shown that diverse glucose responses can be mediated by the HXK1 mutations with little or no catalytic activity (Harrington and Bush, 2003; Moore et al., 2003). However, it poses a major challenge to understand how HXK1 executes its glucose sensing and signaling functions.

Although nuclear localization of HXKs has been observed in mammalian and plant cells (Alvarez et al., 2002; Wilson, 2003; Yanagisawa et al., 2003), their well-defined enzymatic roles in the first step of glycolysis (Kim and Dang, 2005) may have obscured or prevented investigation seeking unconventional/nonglycolytic functions of HXK in the past. We have previously shown that *Arabidopsis* HXK1 can be expressed and detected in the nucleus of maize mesophyll protoplasts (Yanagisawa et al., 2003). Here, we present evidence that endogenous HXK1 resides in the nucleus of intact *Arabidopsis* plants, albeit at minute amount. We hypothesized that HXK1 might mediate glucose sensing and signaling in different cellular compartments with shared or distinct partners. To explore novel mechanisms underlying the nuclear HXK1 actions in diverse glucose responses, we designed sequential screens based on proteomics and the yeast two-hybrid (Y2H) assay to search for the proteins that directly interact with HXK1 in the nucleus. We identified two HXK1 novel partners, VHA-B1 and RPT5B, that have never been previously suspected to play a role in glucose signaling. Similar to HXK1, both VHA-B1 and RPT5B have other well-established functions, for which they work with different partners in the vacuolar H^+-ATPase (V-ATPase/VHA) and proteasome complexes, respectively (Sze et al., 2002; Yang et al., 2004). Only in the nucleus, these three proteins interact and act synergistically to mediate glucose sensing and signaling. Genetic, transgenic, biochemical, and molecular evidence supports an essential role of the novel HXK1 nuclear complex in controlling diverse glucose responses in whole plants.

RESULTS

Identification of HXK1 Unconventional Partners in the Nucleus

To assess the possibility that endogenous glucose sensor HXK1 could be detected in the nucleus of wild-type (WT) *Arabidopsis* plants, we treated ground leaves with the detergent Triton X-100 to solubilize most proteins in the organelles but kept the nuclei intact with Mg^{2+} (Sheen, 1993; Yanagisawa et al., 2003). As expected, the majority of HXK proteins were detected in the detergent-solubilized fraction using the specific antibody for HXK, which was not detected in the *gin2* mutant (Figure 1A) (Jang et al., 1997; Moore et al., 2003). However, relatively small but reproducible amount of HXK was detected together with the major nuclear protein histones in the nuclear fraction (Figure 1A) (Sheen, 1993; Yanagisawa et al., 2003). Protein blot analysis using other specific antibodies against abundant proteins in each fraction confirmed that there was little cross contamination. For example, ribulose 1,6-bisphosphate carboxylase (RBC) was exclusively found in the soluble fraction, whereas histone was present only in the nuclear fraction (Figure 1A). Using a transgenic *gin2* mutant line complemented by the epitope-tagged HXK1-FLAG (Moore et al., 2003), we could also clearly identify HXK1-FLAG in the nuclear fraction using a specific FLAG antibody (Figure 1A).

To initiate an investigation of nuclear HXK1 functions, we designed a six-step procedure to identify proteins that directly interact with HXK1 in the nucleus (Figure 1B and Supplemental Experimental Procedures) and may play important roles in the HXK1-mediated glucose signaling (Jang et al., 1997; Moore et al., 2003). To isolate in vivo protein complexes, the *gin2* transgenic line fully complemented by HXK1-FLAG (Moore et al., 2003) was used to take advantage of the robust immuno-affinity purification system based on FLAG epitope tag (Ho et al., 2002). The first enrichment step by nuclear fractionation was very critical, because the presence of abundant HXK1 proteins outside the nucleus for glucose metabolism and other functions could impede our experimental purpose focused on nuclear glucose signaling (Moore et al., 2003). The specificity of the proteins purified by the FLAG antibody-conjugated agarose was confirmed by the negative control experiments using WT or *gin2* leaves lacking HXK1-FLAG (data not shown). Around 50 visible bands were excised and subjected to tryptic digestion before multiple runs of MALDI-TOF mass spectrometry (MS) and extensive bioinformatics peptide searches (Table S1 and Supplemental Experimental Procedures). To identify the direct HXK1-interacting partners, it was essential to perform the Y2H screen with 48 candidates using HXK1 as bait (Figure 1B and Supplemental Experimental Procedures). The sequential proteomics and Y2H screens identified VHA-B1 and RPT5B as the only two proteins directly interacting with HXK1 (Table S1). The three-way binary interactions among HXK1, VHA-B1, and RPT5B are also demonstrated by Y2H analysis (Figure 1C).

Unexpectedly, VHA-B1 is one of the three expressed isoforms for the B subunit of V_1 complex in V-ATPase (Table S1) (Sze et al., 2002). Plant V-ATPase has been shown to localize in vacuoles, the endoplasmic reticulum, Golgi, small vesicles, and the plasma membrane. All three VHA-B isoforms (VHA-B1, VHA-B2, and VHA-B3) are detected by the proteomic analyses of vacuoles and the tonoplast (Carter et al., 2004; Shimaoka et al., 2004) and presumably serves their traditional and conserved functions in

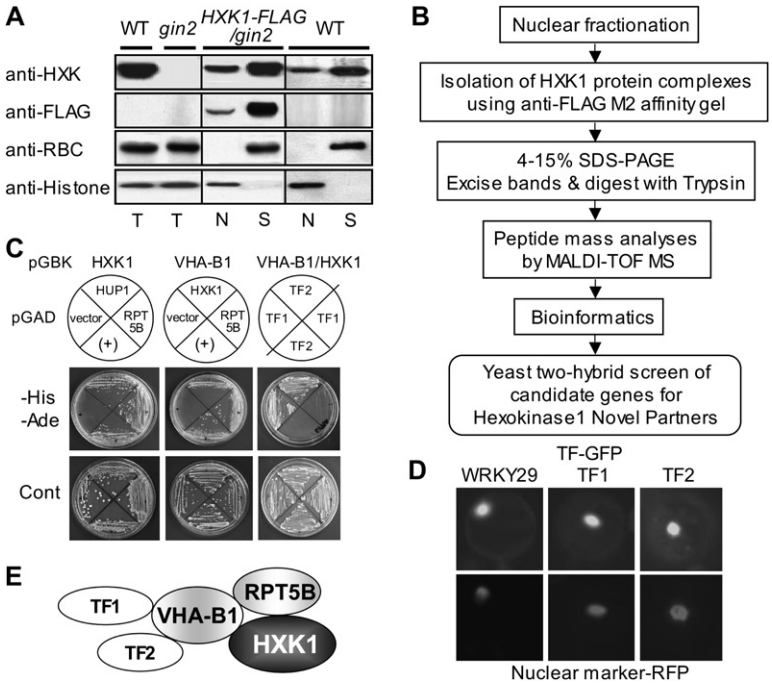

Figure 1. Identification of HXK1 Signaling Partners in the Nucleus

(A) Detection of endogenous HXK1 and epitope-tagged HXK1-FLAG in the nucleus. Total protein extracts (T) from wild-type (WT) and *gin2* mutant were used to show the specificity of antibodies by protein-blot analysis. The same antibodies were used to show that HXK1 was detected in both the nuclear (N) and soluble (S) fractions isolated from the transgenic *gin2* line expressing HXK1-FLAG (*HXK1-FLAG*) and WT. The purity of the nuclear and soluble fractions was demonstrated using specific antibodies against histone and RBC, respectively.

(B) Experimental flow chart for the identification of HXK1partners in the nucleus.

(C) Both VHA-B1 and RPT5B directly interact with HXK1. TF1 and TF2 interact with VHA-B1 but not HXK1. Various cDNAs were cloned into the Y2H vectors and tested on selection plates (−His and −Ade) for protein interactions and on control plates (Cont) for plasmid transformation. Positive control (+, Clontech) was included in each experiment.

(D) TF1-GFP and TF2-GFP are localized in the nucleus. The nuclear red-fluorescent protein (RFP) serves as a nuclear marker in cotransfected protoplasts. WRKY29-GFP is a positive control.

(E) Protein-interacting relations of the nuclear HXK1 protein complex core.

supporting the ATPase activity of the V_1 complexes (Nishi and Forgac, 2002; Sze et al., 2002). The identification of RPT5B as one of the two expressed isoforms of the 19S regulatory particle triple-A ATPase (Table S1) in the conserved base complex of proteasome was also surprising (Rubin et al., 1998; Fu et al., 1999; Yang et al., 2004). The 19S RP is a stable and evolutionarily conserved protein complex found in both cytosol and the nucleus. The six AAA-ATPases in the base serve nonredundant functions in proteasome-dependent or proteasome-independent processes (Rubin et al., 1998; Gonzalez et al., 2002; Yang et al., 2004; Lee et al., 2005). The high resolution of MS analysis allowed the identification of a specific isoform of proteins produced from small gene families (Table S1).

None of the V-ATPase components has previously been reported to be present in the nucleus or to interact with HXK (Nishi and Forgac 2002; Sze et al., 2002). An additional Y2H screen with the 48 candidates using VHA-B1 as bait revealed the direct interaction of VHA-B1, but not HXK1, with two putative transcription factors (At1g50420: SCARECROW-like TF1 and At3g11280: MYB-like TF2) (Figure 1C and Table S1). The putative TFs were only localized to the nucleus visualized by their GFP fusions in *Arabidopsis* mesophyll protoplasts (Figure 1D). The results further support the unconventional interactions and localization of VHA-B1, RPT5B, and HXK1 in the nucleus (Figure 1). No glycolytic enzymes were identified in the nuclear HXK1 protein complexes. In this study, we focused on the detailed analyses of VHA-B1 and RPT5B functions in glucose signaling in *Arabidopsis*.

Specificity of HXK1 and VHA-B1/RPT5B Interactions

VHA-B1 and RPT5B are the unique isoforms of *Arabidopsis* VHA-B and RPT5 that reside in stable protein complexes with well-established partners. To further evaluate the specificity of their interactions with HXK1, we tested the interactions between HXK1 and VHA-B2, VHA-B3, or RPT5A, the closest homolog of VHA-B1 and RPT5B (Fu et al., 1999; Sze et al., 2002; Yang et al., 2004). As shown in Figures 2A and 2B, only the HA-tagged VHA-B1 and RPT5B, but not the HA-tagged RPT5A, VHA-B2 or VHA-B3, were specifically coimmunoprecipitated by the FLAG-tagged HXK1 when transiently expressed in *Arabidopsis* mesophyll protoplasts. VHA-B1 was more efficiently pulled down by HXK1 when RPT5B was coexpressed (Figure 2A), confirming the tight interaction of the three-protein complex revealed independently by the binary Y2H analysis (Figures 1C and 1E). Based on the same coimmunoprecipitation assay, we showed that other key subunits of the V_1 complex in V-ATPase, including VHA-A, VHA-C, and VHA-H encoded by unique genes in *Arabidopsis* (Sze et al., 2002), did not interact with HXK1 (Figures 2B and 2C). The expression of RPT5A, VHA-B2, VHA-B3, VHA-A, VHA-C, and VHA-H proteins in transfected protoplasts was as abundant as VHA-B1 and RPT5B examined using the HA antibody (Figures 2A, 2B,

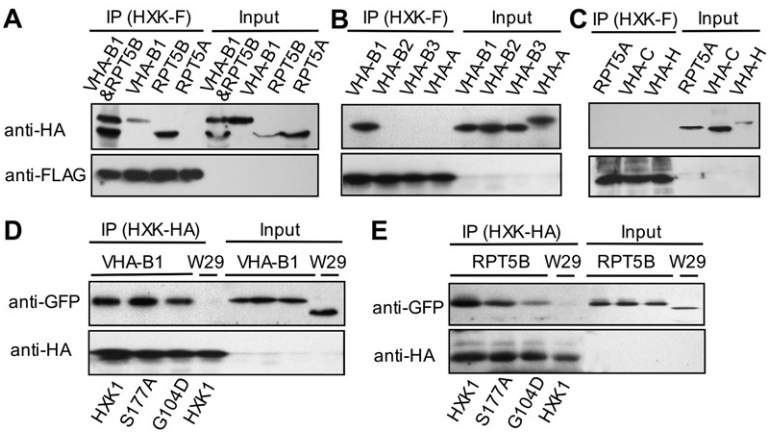

Figure 2. Specific Interactions between HXK1 and Unconventional Partners

(A) HXK1 specifically interacts with VHA-B1 and RPT5B. Each lysate from transfected protoplasts was analyzed by protein-blot analysis after immunoprecipitation (IP, upper panel) with HXK1-FLAG (HXK1-F) (lower panel). After the IP, supernatants were subjected to protein-blot analysis to verify the protein expression (Input, upper panel). RPT5A serves as a negative control.

(B and C) No interactions between HXK1 and other V-ATPase subunits. Two VHA-B1 homologs (VHA-B2 and VHA-B3) and other V-ATPase subunits (VHA-A, VHA-C, and VHA-H) do not interact with HXK1 in the coIP assay.

(D and E) Interaction with VHA-B1 or RPT5B does not require HXK1 catalytic activity. VHA-B1 or RPT5B interacts with WT HXK1 as well as two catalytically inactive HXK1 proteins (S177A and G104D). WRKY29-GFP (W29) serves as a negative control.

and 2C). The data provided additional evidence that the proteomic-based isolation and identification of specific components in the nuclear HXK1 complexes is reliable and consistent with extensive Y2H analyses (Figure 1 and Table S1).

It has been shown previously that two HXK1 mutations with no ($HXK1^{S177A}$) or little ($HXK1^{G104D}$) catalytic activity can complement most HXK1 functions as a glucose sensor in *Arabidopsis* (Moore et al., 2003). To see whether interactions with HXK1 required its enzyme activity, coimmunoprecipitations were examined between VHA-B1 or RPT5B and WT HXK1, $HXK1^{S177A}$ or $HXK1^{G104D}$. To facilitate the identification of specific proteins by epitope tag and size differences, HXK1 variants were tagged with HA but VHA-B1 and RPT5B were tagged with GFP (Chiu et al., 1996). A nuclear WRKY29-GFP (Asai et al., 2002) was used as a negative control in the coimmunoprecipitation with HXK1-HA. VHA-B1 interacted with WT and catalytically inactive HXK1 equally well, and RPT5B appeared to interact more effectively with the WT HXK1 (Figures 2D and 2E). Nevertheless, the interactions potentially important for glucose signaling did not require the glucose phosphorylation activity of HXK1.

Unique Interaction of Endogenous HXK1, VHA-B1, and RPT5B in the Nucleus

To provide further evidence for the novel interactions of HXK1 with VHA-B1 and RPT5B in the nucleus in vivo, we used the HXK antibody to pull down the endogenous nuclear HXK1 protein complexes from the leaves of WT plants. Protein blot analyses with specific antibodies verified that only endogenous VHA-B1 and RPT5B, but not VHA-A (Sze et al., 2002) or RPT1 (Yang et al., 2004), could be copurified with HXK1 in the nucleus (Figure 3A). Furthermore, the VHA-B antibody was used to show the coimmunoprecipitation of endogenous VHA-B1 with HXK1 and RPT5B in the nucleus but not VHA-A or RPT1 (Figure 3B).

As positive controls for endogenous protein interactions in other compartments, the VHA-B and RPT5 antibodies were applied to the detergent-solubilized fraction. The results demonstrated the well-established partnerships for VHA-A and VHA-B in the V_1 complex of V-ATPase (Sze et al., 2002) and for RPT1 and RPT5 in the 19S RP ATPase complexes (Fu et al., 1999; Yang et al., 2004) (Figure 3C). Interestingly, HXK1 did not coimmunoprecipitate with endogenous VHA-B1 or RPT5B outside the nucleus (Figure 3C), confirming the nuclear-specific interactions among HXK1, VHA-B1, and RPT5B. These results provide strong evidence for the in vivo interactions of VHA-B1,

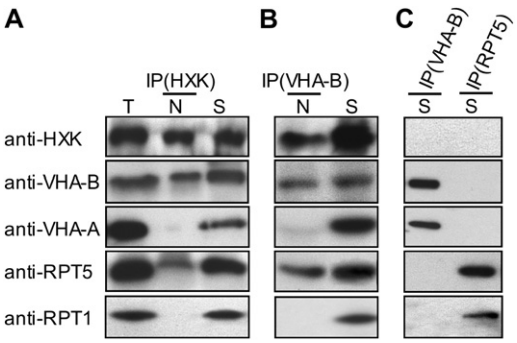

Figure 3. In Vivo Interaction Analyses of Endogenous HXK1, VHA-B1, and RPT5B

(A) HXK1 interacts specifically with VHA-B1 and RPT5B in the nucleus in vivo. The HXK antibody was used for IP. Each protein is detected with specific antibody in total lysates (T), nuclear (N), or soluble (S) fraction.

(B) Endogenous HXK1 and RPT5B coimmunoprecipitated with the endogenous VHA-B in the nucleus of WT plants. The VHA-B antibody was used for IP.

(C) VHA-B1 and RPT5B are detected in distinct protein complexes of the soluble (S) fraction of WT plants. VHA-B and RPT5 antibodies were used for IP.

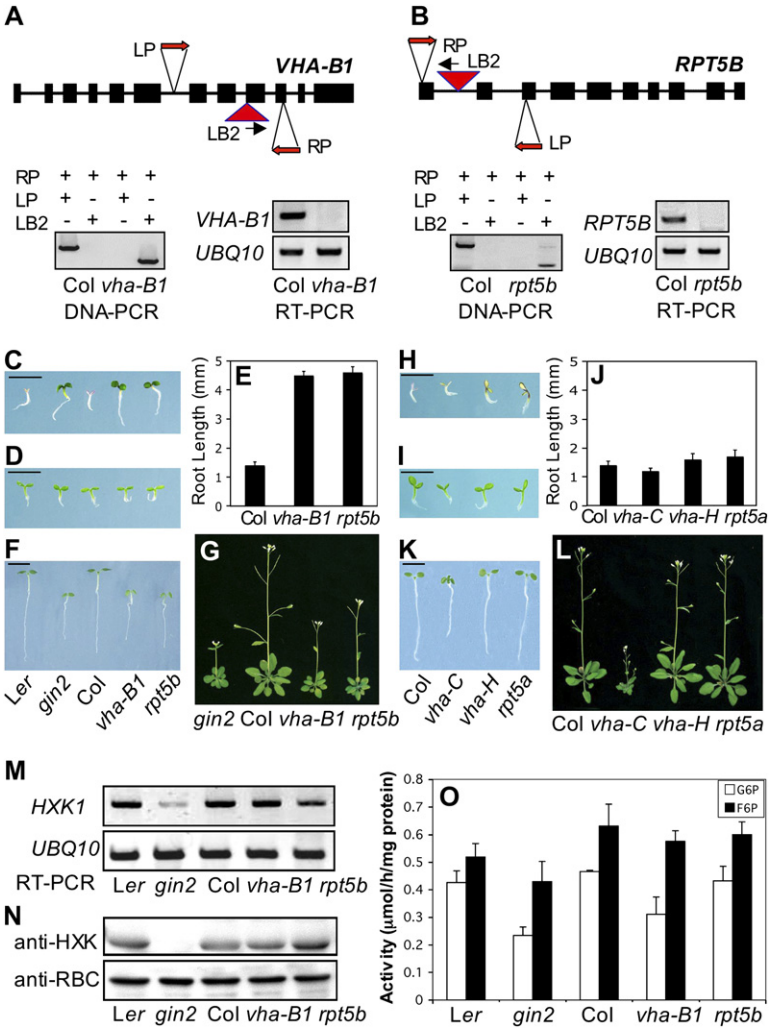

Figure 4. The *vha-B1* and *rpt5b* Mutants Are Glucose Insensitive

(A and B) Molecular analysis of the *vha-B1* and *rpt5b* mutants, respectively. The T-DNA insertion sites and primer (LP, RP, and LB2) locations are indicated. Expression of *VHA-B1* and *RPT5B* transcripts was shown by RT-PCR with gene-specific primer sets. *UBQ 10* serves as an internal control.

(C) The *gin2*, *vha-B1*, and *rpt5b* mutants show similar insensitivity to glucose-mediated developmental arrest observed in WT (L*er* or Col). Scale bar, 5 mm.

(D) WT, *gin2*, *vha-B1*, and *rpt5b* mutants show similar growth on mannitol medium.

(E) Quantitative analysis of glucose repression of root elongation in WT, *vha-B1*, and *rpt5b*. Each measurement represents the mean of primary root length with an error bar indicating standard deviation of 20 samples.

(F) The *gin2*, *vha-B1*, and *rpt5b* mutants exhibit seedling growth retardation when compared with WT (L*er* or Col) under the low-light and low-nutrient condition.

(G) The *vha-B1* and *rpt5b* mutants display similar growth defects as *gin2* in adult plants under high light.

(H) Analysis of V-ATPase and 19S RP mutants. The *vha-C*, *vha-H*, and *rpt5a* mutants display similar glucose-mediated developmental arrest as WT (Col).

(I) Similar seedling growth of *vha-C*, *vha-H*, and *rpt5a* on mannitol.

(J) Quantitative analysis of glucose repression of root elongation in *vha-C*, *vha-H*, and *rpt5a*. Each measurement is the same as described in (E).

(K) Seedling-growth analysis of *vha-C*, *vha-H*, and *rpt5a* under low-light and low-nutrient condition.

(L) Analysis of adult-plant growth in *vha-C*, *vha-H*, and *rpt5a* under high light. The *vha-C* mutant displays growth defects distinct from those in *gin2, vha-B1*, and *rpt5b*.

(M) The *vha-B1* and *rpt5b* mutants do not alter *HXK1* transcript expression.

(N) Normal HXK1 protein levels in *vha-B1* and *rpt5b*. HXK1 was detected by protein-blot analysis using HXK antibody. RBC serves as a protein-loading control.

(O) Analysis of glucose (G6P) and fructose (F6P) phosphorylation activities in *vha-B1* and *rpt5b*. Values are means of triplicate measurements with error bars representing standard deviation. The experiments were repeated twice with similar results.

RPT5B, and HXK1 in a novel nuclear entity independent of their traditional protein complexes.

Genetic Analyses of VHA-B1 and RPT5B Functions in Glucose Signaling

To elucidate the physiological functions of VHA-B1 and RPT5B in the HXK1-mediated glucose signaling in whole plants, we obtained loss-of-function *vha-B1* and *rpt5b* mutants from the T-DNA insertion resource (Figures 4A and 4B and Supplemental Experimental Procedures) (Alonso et al., 2003). In the homozygous lines, no *VHA-B1* and *RPT5B* transcript expression could be detected by reverse transcriptase-dependent polymerase chain reaction (RT-PCR) (Figures 4A and 4B). We performed phenotypic analyses of *vha-B1* and *rpt5b* mutants using diverse bioassays well established for the HXK1 mutant *gin2* (Moore et al., 2003). Similar to *gin2*, both *vha-B1* and *rpt5b* were insensitive to the high-glucose repression of cotyledon expansion, chlorophyll accumulation, true-leaf development, and root elongation (Figures 4C and

4E). The response was specific to glucose but not to osmolarity changes because WT, *gin2*, *vha-B1*, and *rpt5b* seedlings displayed the same phenotypes on the mannitol medium (Figure 4D). It has previously been demonstrated that not only is HXK1 responsible for growth repression in the presence of high exogenous glucose but it is also essential for growth promotion by physiological levels of glucose in seedlings and adult plants. For example, leaf, root, and inflorescence growth is positively correlated with endogenous glucose levels controlled by light intensity (Moore et al., 2003). Remarkably, both the *vha-B1* and *rpt5b* mutants displayed the same seedling- (Figure 4F) and adult-plant (Figures 4G and S1) growth defects observed in the glucose-insensitive *gin2* plants (Moore et al., 2003). Together with evidence for direct interactions of HXK1, VHA-B1, and RPT5B in the nucleus, these genetic and phenotypic analyses support the idea that VHA-B1 and RPT5B act together with HXK1 in glucose sensing and signaling pathways.

To obtain genetic evidence for the uncoupling of glucose signaling from the conserved V-ATPase and proteasome functions, we evaluated other *Arabidopsis vha* and *rpt* mutants. It has recently been reported that *vha-A* and *vha-E1* mutants are lethal, implying the essential role of V-ATPase in embryogenesis and male gametophyte development (Dettmer et al., 2005; Strompen et al., 2005). However, *gin2*, *vha-B1*, and *rpt5b* null mutants were not lethal as the typical V-ATPase mutants. Another V-ATPase mutant *det3/vha-C* was sensitive to glucose (Figures 4H and 4J) and displayed dwarf and branching morphology distinct from *gin2*, *vha-B1*, and *rpt5b* (Figures 4G and 4L). The *vha-H* and *rpt5a* null mutants were also glucose sensitive and did not show any visible phenotypes (Figures 4H–4L). In *Arabidopsis*, three VHA-B and two RPT5 proteins likely play overlapping functions for the conserved VHA (Sze et al., 2002; Carter et al., 2004; Shimaoka et al., 2004) and AAA-ATPase activity in the proteasome (Fu et al., 1999; Yang et al., 2004), respectively.

To further support the uncoupling of glucose signaling from glucose metabolism in *Arabidopsis*, we showed that the mutant phenotypes of *vha-B1* and *rpt5b* were not the consequences of defects in *HXK* transcript expression (Figure 4M), protein stability (Figure 4N), or glucose/fructose phosphorylation activities (Figure 4O). Currently, we do not have an explanation for the slightly reduced glucose phosphorylation activity observed in *vha-B1* (Figure 4O). However, we have demonstrated that even the more severe reduction of glucose/fructose phosphorylation activities in *gin2* is not critical for glucose signalling because the catalytically inactive HXK1^{S177A} is sufficient to restore glucose responses (Moore et al., 2003). Since we only obtained one allele each for the *vha-B1* and *rpt5b* mutants, it was important to show that the WT *VHA-B1* and *RPT5B* genes could eliminate all the glucose-signaling defects in multiple and independent transgenic lines. We selected the transgenic lines expressing a similar level of *VHA-B1* and *RPT5B* transcripts as those in WT plants for phenotypic analyses (Figures 5A and 5B). Compared to WT

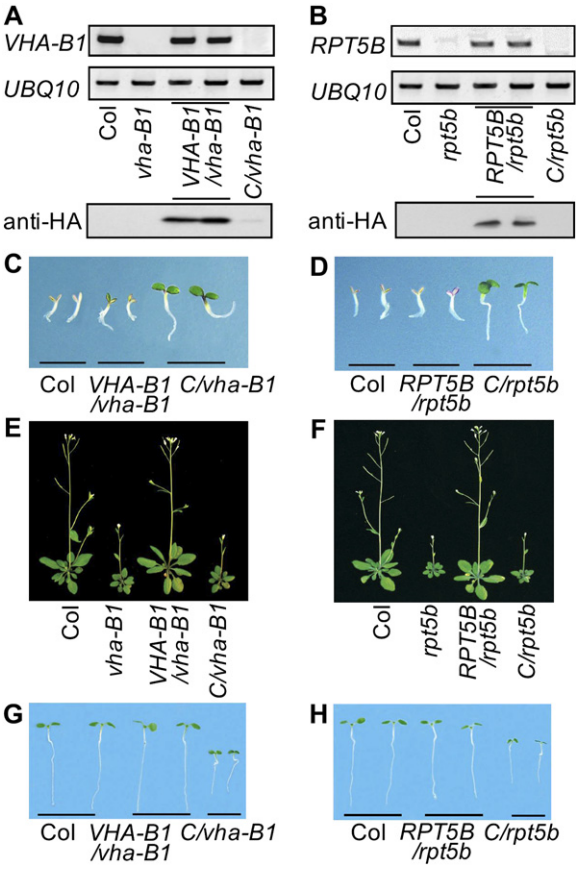

Figure 5. Complementation Analyses of *vha-B1* and *rpt5b* Mutants

(A and B) Analysis of *VHA-B1* and *RPT5B* transcripts and protein expression in complemented lines. WT (Col) and transgenics with the empty vector (*C/vha-B1* or *C/rpt5b*) were used as controls.

(C and D) Analysis of glucose-mediated developmental arrest.

(E and F) Analysis of adult-plant growth under high light.

(G and H) Analysis of seedling growth under low-light and low-nutrient condition.

plants or transgenic lines transformed with only the empty binary vector, multiple independent transgenic lines expressing WT *VHA-B1* or *RPT5B* RNAs and VHA-B1-HA or RPT5B-HA proteins successfully complemented the glucose-specific phenotypes in the *vha-B1* or *rpt5b* mutant plants (Figures 4C, 4F, 4G, and 5C–5H).

The Nuclear HXK1 Complex Controls Glucose Repression on Specific Chromatin

One conserved and central glucose response in diverse plant species is the transcription repression of photosynthetic genes by glucose (Sheen, 1990; Koch 1996; Jang et al., 1997; Smeekens, 2000; Coruzzi and Bush, 2001; Moore et al., 2003). Similar to *gin2*, both *vha-B1* and *rpt5b* showed insensitivity to the specific glucose repression of chlorophyll *a/b* binding protein (*CAB*) and carbonic anhydrase (*CAA*) gene expression. The same concentration of mannitol did not cause gene repression and served

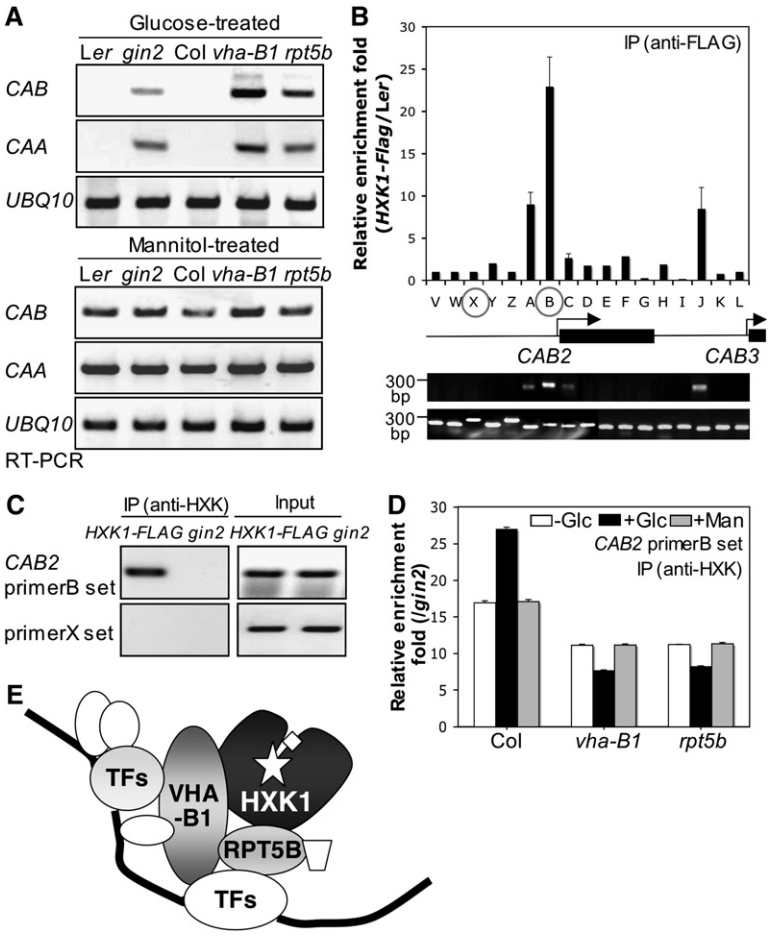

Figure 6. VHA-B1 and RPT5B Are Essential for Glucose-Dependent Gene Regulation Mediated by HXK1

(A) The *gin2*, *vha-B1*, and *rpt5b* mutants are insensitive to glucose repression of *CAB* and *CAA*. *UBQ10* expression is not affected by glucose and serves as a control. Mannitol does not repress *CAB* and *CAA*.

(B) The nuclear HXK1 protein complex binds directly to specific target gene *CAB2* promoters. Genomic DNA obtained by ChIP using FLAG antibody in the plants expressing HXK1-FLAG was analyzed by quantitative PCR (qPCR) and normalized based on the WT (*Ler*) background lacking HXK1-FLAG. Primer sets A, B, and J produced specific PCR fragments (upper gel). All PCR primer sets generated predicted DNA fragments with genomic DNA as controls (lower gel). Values are means of triplicate measurements with error bars representing standard deviation. The experiments were repeated twice with similar results.

(C) ChIP-qPCR analysis using HXK antibody. The *CAB2* promoter chromatin is enriched in the plants expressing *HXK1-FLAG* but not in *gin2* plants. PCR products before ChIP are shown as input controls.

(D) Loss of VHA-B1 or RPT5B compromises the binding of the HXK1 complex to the *CAB2* promoter. ChIP-qPCR experiments were performed in WT (Col), *vha-B1*, and *rpt5b* mutant seedlings using the HXK antibody without or with 2% glucose or mannitol treatment for 6 hr. Values are normalized based on those obtained from *gin2* seedlings lacking HXK1 with equal input of genomic DNA and are means of triplicate measurements with error bars as described in (B).

(E) Model of nuclear glucose sensing and signaling through HXK1, VHA-B1, and RPT5B. Putative TFs can interact directly with VHA-B1 or with both VHA-B1 and RPT5B and mediate glucose regulation of specific target gene transcription by the HXK1 complexes binding to the chromatin. VHA-B1 and RPT5B connect other proteins to relay HXK1 sensing and signaling. The precise protein-protein interacting sites are unknown. Line: chromatin; star: glucose; square: ATP.

as a control (Figure 6A). The result further supports the synergistic action of the three partners in nuclear glucose signaling. To see whether the nuclear HXK1 complex binds directly to the specific target gene promoters, we performed chromatin immunoprecipitation (ChIP) experiments using FLAG antibody to pull down the nuclear HXK1-FLAG protein complexes cross-linked to the endogenous genomic DNA in vivo. The genomic DNA was isolated from the chromatin and analyzed by quantitative PCR using a set of primers scanning through about 4.7 kb region of the *CAB2* gene (Figure 6B). As a positive control, all primer sets generated predicted PCR products with *Arabidopsis* genomic DNA isolated from both *Ler* (Figure 6B) and Col (data not shown). Consistent with the location of functional regulatory *cis* elements upstream of the *Arabidopsis CAB2* coding region, the HXK1 complex bound specifically to the 5′-272 bp region of the *CAB2* promoter (A-B region) containing DtRE, CUF-1, CCA1, and CGF-1 motifs (Figure 6B) (Chiu et al., 1996; Maxwell et al., 2003). We also identified another specific HXK1 complex binding site in the promoter of *CAB3*, another glucose repressed target gene. Interestingly, CUF-like elements were also present in the *CAB3* promoter. The result further verifies the nuclear function of HXK1 and is consistent with previous studies suggesting that the conserved and short *CAB* promoters in maize and *Arabidopsis* are the targets for glucose repression (Sheen, 1990, 1993; Chiu et al., 1996; Maxwell et al., 2003). Further studies will reveal the precise *cis* elements, TFs, and chromatin modifications modulated by the HXK1 complex in mediating glucose repression.

To substantiate the specific binding of HXK1 complex on the *CAB2* promoter in vivo, we repeated the ChIP-PCR experiments using the HXK antibody (Figure 6C). The specificity of the data was reinforced by a negative control using *gin2* lacking HXK1 (Figure 4N), which yielded only background PCR products (Figure 6C). To examine the role of VHA-B1 and RPT5B in the binding of the nuclear HXK1 complex to the *CAB2* promoter, ChIP was performed in the *vha-B1* and *rpt5b* mutants using the HXK

antibody without or with exogenous glucose. The *gin2* mutant was used for the background control. By quantitative PCR analysis, it was obvious that both VHA-B1 and RPT5B are important for the HXK1 complex binding to the *CAB2* promoter (Figure 6D), especially in the presence of 2% exogenous glucose when *CAB2* expression is repressed (Figure 6A). However, lacking either VHA-B1 or RPT5B only diminished but not abolished the chromatin binding activity (Figure 6D). Although both VHA-B1 and RPT5B are absolutely required for the glucose repression of *CAB2* (Figure 6A), they may have overlapping functions linking the nuclear HXK1 to specific target gene promoters (Figure 6E). HXK1, VHA-B1, and RPT5B do not contain recognizable DNA binding motifs/domains, but VHA-B1 and RPT5B could directly interact with putative TFs identified during the proteomic analysis of the nuclear HXK1 protein complex (Figures 1C and 6E and Table S1) (Y.-H.C., S.-D.Y., and J.S.; unpublished data). VHA-B1 and RPT5B are likely the key mediators of glucose repression connecting HXK1 to DNA binding TFs that target to specific genes on the chromatin (Figure 6E).

DISCUSSION

Applying a combination of nuclear proteomic and Y2H screens, we have identified two unconventional partners of the HXK1 glucose sensor in the nucleus of *Arabidopsis*. Surprisingly, VHA-B1 and RPT5B also have well-established functions in V-ATPase and the 19S proteasome subcomplex, respectively. However, only VHA-B1 and RPT5B, but not their closely related isoforms, are found in the nuclear HXK1 protein complexes (Figures 1 and 3 and Table S1) and directly interact with HXK1 in vivo (Figures 2 and 3). Proteomic analysis of mitochondrial proteins involved in glycolysis has also identified HXK1 and HXK2 but not VHA-B1 and RPT5B (Giege et al., 2003; Heazlewood et al., 2004). The novel roles of VHA-B1 and RPT5B in nuclear glucose signaling are conclusively demonstrated by the characterization of loss-of-function *vha-B1* and *rpt5b* mutants showing similar whole plant phenotypic and glucose response defects found in *gin2* and verified by complementation analyses (Figures 4, 5, and 6). Finally, glucose-dependant gene repression and ChIP experiments in intact plants suggest that HXK1 and VHA-B1/RPT5B are in contact with specific target gene promoters and directly regulate glucose-mediated transcription repression (Figure 6). The studies support a novel concept that a key metabolic enzyme can form complexes with other conserved proteins to play unique roles and directly control gene expression in the nucleus, thus uncoupling its signaling activities from metabolism.

Integrative Approaches in Signal Transduction

Genetic screens for sugar-signaling mutants in the past decade have mainly yielded multiple alleles of overlapping sets of genes involved in hormone biosynthesis and signaling in *Arabidopsis*. The efforts have revealed the intimate and complex relationships between glucose and plant hormones (Smeekens, 2000; Gibson, 2005; Rolland et al., 2006). However, the stringent screening conditions at high-sugar concentrations might have caused complex effects, which enriched the isolation of hormone mutants with strong phenotypes (Rolland et al., 2006). The availability of the *Arabidopsis* genome sequences, sensitive MS, and effective bioinformatic tools have enabled the multistep proteomic-based screens to discover novel functions of proteins in the core of the glucose sensing and signaling complex. It is intriguing that HXK1, VHA-B1, and RPT5B all have well-established and conserved functions in metabolism and key cellular processes (Sze et al., 2002; Harrington and Bush, 2003; Moore et al., 2003; Yang et al., 2004). Standard biochemical purification of HXK1 relying on quantity or enzyme activity without the nuclei-purification step would have missed the important functional complex representing only a minute proportion of total HXK1 in specific cellular locals. The proteomic-based integrative approaches used in this study overcame inherent limitation of each individual method. Comprehensive molecular, cellular, genetic, and genomic evidence is critical in determining the specificity and functions of glucose-signaling components. The analysis of the proteins directly interacting with the glucose sensor HXK1 in a defined compartment ensured the specificity of their actions in the glucose-signaling pathways. It may be informative to reevaluate existing loss-of-function mutants with distinct defects in conserved proteins, especially for the members of huge protein complexes. Some of them may have additional and unrelated functions similar to VHA-B1 and RPT5B. Our work provides new evidence for the emerging complexity that the same signaling components could function in multiple complexes and subcellular compartments for diverse functions.

Unconventional Metabolic Enzyme Sensors and Transcription Regulators

Genes encoding metabolic enzymes have been mostly excluded from the research on signal-transduction pathways in the past. Recent serendipitous studies and unbiased proteomic screens have uncovered a few examples suggesting that some enzymes have more than the traditional metabolic roles (Hall et al., 2004; Kim and Dang, 2005). For example, the characterization of a multicomponent coactivator complex essential for the histone *H2B* transcription in S phase identifies a nuclear glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Zheng et al., 2003). As the histone *H2B* promoter is modulated by NAD^+/NADH, GAPDH may sense and transduce cellular redox state to modulate transcription. In yeast, galactokinase binds directly to Gal80 and controls Gal4 activation in response to galactose and ATP (Zenke et al., 1996). Through screening a yeast proteome microarray using genomic DNA, the mitochondria enzyme Arg5,6 involved in arginine/ornithine biosynthesis has been identified. ChIP experiments confirm the association of Arg5,6 with specific nuclear and mitochondria loci, and the deletion of Arg5,6 affects transcript levels of target genes. Although

Arg5 has been detected in mitochondria, its abundance in the nucleus is too low to be observed by indirect immunofluorescence (Hall et al., 2004). Our studies based on the characterization of *vha-B1* and *rpt5b* mutants and ChIP experiments have provided the first evidence that the nuclear HXK1 protein complex with VHA-B1 and RPT5B are essential for glucose-mediated repression of a specific target gene *CAB2* promoter. Since the nuclear HXK1 complex does not contain any other enzymes involved in glycolysis and VHA-B1 and RPT5B interact with catalytically inactive HXK1 (Figures 2D and 2E), glucose metabolism is most likely not required in HXK1-mediated transcription repression. It is conceivable that similar to sensor or receptor binding to specific ligands, metabolic enzymes can act like sensors by binding to specific signals or metabolites and alter their conformation and/or protein-protein interactions in complexes to initiate signal transduction. It is plausible that many more enzymes can serve as sensors or transcription regulators directly linking metabolic signals to the central signaling networks.

Glucose Sensing and Signaling Mechanisms and Functions

The observation that *gin2*, *vha-B1*, and *rpt5b* mutants shared similar adult-plant phenotypes supports the model that the three proteins work synergistically to promote vegetative and reproductive plant growth correlated with external light intensity and internal glucose levels (Figures 1, 3, and 4). Interestingly, the same protein complex is also critical for the developmental arrest in seedlings as well as *CAB* and *CAA* repression in response to high glucose levels (Figures 4, 5, and 6). Preliminary global gene expression profiling has indicated that HXK1 controls glucose-mediated expression of many genes, which are insensitive to glucose in the *gin2* mutant (J.S., unpublished data). Future microarray experiments in *gin2*, *vha-B1*, and *rpt5b* will reveal the range of genes that are repressed or activated by glucose in different tissues under various growth conditions.

The studies on mammalian HXKs and GK have emphasized their metabolic functions (Wilson, 2003; Dentin et al., 2004). In plants, both glucose promotion of growth and various glucose-repression responses are mediated by HXK1 without its catalytic activity (Moore et al., 2003). The facts that VHA-B1 and RPT5B have no direct roles in glycolysis and *rpt5b* mutant retains WT level of glucose/fructose-phosphorylation activities (Figure 4O) but the same spectrum of *gin2* phenotypes (Figures 4 and 5) provide further evidence for the uncoupling of glucose signaling from metabolism. It appears that HXK1 and VHA-B1/RPT5B form a distinct, multimeric protein complex in the nucleus for nonenzymatic functions of glucose sensing and signaling. A high level of glucose signals does not appear to be required for the synthesis of leaf HXK1 protein, nuclear localization, interactions with VHA-B1 and RPT5B, and even its binding to the target gene promoter measured by ChIP in vivo (Figures 1, 2, 3, and 6). However, it is difficult to completely deplete glucose in the active cells of whole plants. By altering external glucose levels or light intensity, reproducible glucose responses can be observed and quantified (Moore et al., 2003). Both VHA-B1 and RPT5B are absolutely required for the glucose repression *CAB2* and support the direct binding of the HXK1 complex on the *CAB2* promoter, especially at high glucose levels (Figure 6).

The mechanism of VHA-B1 actions in HXK1-mediated glucose signaling is not fully understood. In the V_1 complex, VHA-B interacts with many partners, including VHA-A, VHA-C, VHA-E, and perhaps VHA-G (Nishi and Forgac, 2002; Sze et al., 2002). VHA-B1 may be recruited to the nuclear HXK1 complexes to serve as an anchoring protein for the glucose-signaling process connecting HXK1 and RPT5B to other components. Putative TFs identified in the nuclear HXK1 complexes so far interact directly with VHA-B1 and RPT5B or both, but not HXK1 (Figures 1C and 6E) (Y.-H.C., S.-D.Y., and J.S.; unpublished data). It is likely that HXK1 with a conserved glucose binding site acts as a sensor and responds directly to glucose signals. The glucose-induced conformation change of HXK1 could then alter the activity of VHA-B1 and RPT5B in their interactions or regulation of multiple protein partners (Figure 6E). Future research will be required to elucidate the precise mechanism of HXK1 and VHA-B1/RPT5B actions and the roles of other components in the HXK1 protein complexes in glucose signaling.

Recent studies have uncovered the nonproteolytic roles of 19S RP in RNA polymerase II-dependent transcription for yeast promoters. All six AAA-ATPases of the 19S RP base appear to be required for transcription activation (Gonzalez et al., 2002; Lee et al., 2005). It will be interesting to determine the new functions of 19S RP in transcription activation of specific gene promoters in plants and mammals. However, the unique role of RPT5B in HXK1-mediated glucose repression may not need the intact 19S RP base. Whether the yeast and the mammalian orthologs of RPT5 have unconventional activities is currently unknown and deserves investigation.

Glucose is a central regulator of physiology, metabolism, growth, and gene expression from yeast to mammals and plants. Although only a small fraction of *Arabidopsis* HXK1 is present in the nucleus, its function is indispensable for glucose-dependent gene expression and plant growth and development. Our findings have opened new possibilities for examining novel functions of HXKs in the nucleus and in other subcellular compartments in eukaryotes. It is also a new challenge to elucidate how HXK1 and VHA-B1/RPT5B are targeted into the nucleus to assemble with other components of the glucose-sensor complexes for the novel and essential functions.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

The plants for proteomic analyses were grown on soil at 23°C for 18 days under 75 μmol/m^2/s and a 13 hr photoperiod. For glucose-repression assays, seedlings were grown on 5% glucose or mannitol MS

medium plates for 4 days under constant light. For the high light (240 μmol/m^2/s) growth assay, plants were grown on soil for 25 days under a 16 hr photoperiod. For the low-nutrient and low-light assay, seedlings were grown on 0.2% glucose and 1/10 MS medium for 8 days under constant dim light (15 μmol/m^2/s). For gene expression and ChIP analyses, seedlings were grown in liquid medium with 0.2% glucose and 1/10 MS medium for 7 days before treating with 2% glucose or mannitol for 6 hr. PCR was carried out with cDNA made with 25 ng total RNA using oligo-dT and SuperScript II Reverse transcriptase (RT) (Invitrogen).

Isolation and Identification of the HXK1 Protein Complex Components

The HXK1 protein complex was isolated from about 100 g of 18 days old plants expressing HXK1-FLAG that readily complements the *gin2* mutant in glucose responses (Moore et al., 2003). After stringent Triton X-100 lysis and nuclear fraction enrichment (Sheen, 1993), the HXK1 protein complexes were obtained by immunoaffinity purification using the FLAG-antibody-conjugated agarose (Sigma) (Ho et al., 2002). WT and *gin2* were used as negative controls to show the specificity of the FLAG tag antibody. The eluted proteins (0.5 μg) using the FLAG peptides were separated by SDS-PAGE (Criterion Tris-HCl 4%–15% Linear Gradient gel, Bio-Rad), silver stained (Invitrogen), and excised individually before washing, dehydrating, trypsinizing (Promega) and analyzed by MALDI-TOF MS and three computer programs (MS-FIT, Peptident, and MOWSE). The experiments were repeated three times, and the details are described in Supplemental Experimental Procedures.

Protoplast Transient Expression and Protein-Blot Assays

Arabidopsis mesophyll protoplasts (20,000/sample) isolated from the *HXK-FLAG* plants were transfected with constructs expressing HA-tagged VHA-B1, RPT5B, VHA-A, VHA-B2, VHA-B3, VHA-C, VHA-H, or RPT5A. Protoplasts lysates were collected at 6 hr after DNA transfection and immunoprecipitated with HXK1-FLAG using FLAG antibody (Sigma) and protein A-agarose (Roche). The proteins from immunoprecipitation were then detected by protein-blot analysis using an anti-HA antibody (Roche). Transiently expressed proteins, including the HA-tagged HXK1, S177A, or G104D and GFP-tagged VHA-B1, RPT5B, or WRKY29, in *gin2* protoplasts were coimmunoprecipitated using the HA antibody and detected by protein-blot analysis using the GFP antibody (Roche). Endogenous HXK1 proteins in the WT (*Ler* or Col-0), *gin2*, *vha-B1*, and *rpt5b* were detected by protein-blot analysis using a polyclonal HXK antibody (Jang et al., 1997). Antibodies for VHA-A and VHA-B were gifts from Dr. H. Sze (Ward et al., 1992). RPT5 and RPT1 antibodies were purchased from BioMol.

ChIP

About 10 g of leaf tissues from 18 days-old HXK-FLAG expressing plants, WT, or *gin2* were used for ChIP with the anti-FLAG (Sigma) or anti-HXK antibody. The crosslink and sonication time and conditions were as described (Johnson et al., 2003). The ChIP results were similar using leaf tissues or seedlings.

Supplemental Data

Supplemental Data include one figure, one table, and experimental procedures and can be found with this article online at http://www.cell.com/cgi/content/full/127/3/579/DC1/.

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