Molecular characterization of HEXOKINASE1 in plant innate immunity

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Abstract

Hexokinase1 (HXK1) is an Arabidopsis glucose sensor that has a variety of roles during plant growth and development, including during germination, flowering, and senescence. HXK1 also acts as a positive regulator of plant immune responses. Previous research suggested that HXK1 might influence plant immune responses via responses to glucose. Plant immune responses are governed by two main pathways: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI involves the recognition of Pathogen-Associated Molecular Patterns (PAMPs) and leads to increased callose formation and accumulation of pathogenesis response (PR) proteins. ETI acts in response to effectors secreted by Gram-negative bacteria. During ETI, the membrane-localized protein RPM1-interacting protein 4 (RIN4) becomes phosphorylated in response to interactions with effectors and mediates the downstream response. In this study, the effects of glucose on plant immune responses against infection with Pseudomonas syringae pv. tomato DC3000 and other P. syringae strains were investigated in the presence and absence of HXK1. Infiltration of leaves with glucose prior to infection led to decreases in bacterial populations and reductions in disease symptoms in wild-type Arabidopsis plants, indicating that glucose plays a role in plant immunity. Both PTI and ETI responses were affected. However, these effects were not observed in a hxk1 mutant, indicating that the effects of glucose on plant immune responses were mediated by HXK1-related pathways.

Keywords: Effector-triggered immunity, Glucose, Hexokinase, PAMP-triggered immunity, Plant immunity

Introduction

Sugar metabolism in plants is a critical and complex process that involves glycolysis, the tricarboxylic acid (TCA) cycle, and pentose phosphate pathways. Hexokinases (HXK) play key roles in sugar metabolism through phosphorylation of glucose to glucose-6-phosphate. The HXK family contains six members: HXK1, HXK2, HXK3, HKL1, HKL2, and HKL3 [1]. HXK1 is a multifunctional protein that is involved in sugar metabolism and signaling. An Arabidopsis hxk1 mutant exhibited delayed flowering and senescence as well as smaller leaves and root systems [2]. HXK1 was shown to affect the concentration of glucose in seedlings, and the absence of HXK1 significantly suppressed the effects of glucose [3].

Another HXK family member, HXK2, is involved in plant immunity. Overexpression of HXK2 led to enhanced plant resistance to pathogens and was correlated with elevated H2O2 production and expression of defensive genes [4]. HXK1 and HXK2 share similar features: both proteins have several functions, one of which is sugar-sensing in Arabidopsis [5].

Plants activate their innate immune systems via two pathways [6]. The first line of activation is PAMP-triggered immunity (PTI), which involves the recognition of Pathogen-Associated Molecular Patterns (PAMPs) by membrane-localized Pattern Recognition Receptors (PRRs) [7]. In the very early stages of the PTI response (within 1–5 min), PAMPs such as flagellin and EF-Tu are activated by PRRs, namely FLS2 and EFR [8, 9], after
which the FLS-BAK1 complex forms within 2 min [10, 11]. Ion fluxes, oxidative bursts, and protein phosphorylation also occur during this stage. In the next stage of the response (5–30 min), PTI induces ethylene biosynthesis, receptor endocytosis, and gene activation. These early responses lead to callose deposition and seedling growth inhibition over a longer timescale (hours-days) [12]. Subsequently, the accumulation of PR proteins instigates Systemic Acquired Resistance (SAR), which expands the local immune response of the plant to act against a broad spectrum of pathogens [13].

Effector triggered immunity (ETI) is the second line of activation of the plant immune system [6]. ETI acts in response to effector proteins secreted by the type III secretion system (TTSS) in Gram-negative plant-pathogenic bacteria [14]. Receptor (R) proteins which contain both Nucleotide-Binding (NB) and Leucine-Rich Repeat (LRR) domains can be triggered by direct interactions with their corresponding avirulence (Avr) effectors [15, 16] or indirectly via detection of the action of an Avr effector on its target [17, 18].

The hypersensitive response (HR), a type of plant resistance response, induces programmed cell death at infection sites and inhibits pathogen growth [19]. Arabidopsis protein RIN4 is a well-characterized component of this type of resistance response, and can be explained by the guard hypothesis [6]. RIN4 is a small protein that localizes to the plasma membrane alongside several guard proteins, including RPM1 (resistance to *P. syringae pv. maculicola*) and RPS2 (resistance to *P. syringae 2*) [20, 21]. Bacterial type III effector protein AvrRpm1 acts via RIPK and related kinases to mediate phosphorylation of RIN4 and thereby activate RPM1 [22]. AvrRpt2 is a cysteine protease which cleaves RIN4 at two sites, producing three fragments of 15.9 kDa, 6.4 kDa, and 1.2 kDa [23]. Degradation of RIN4 activates RPS2 and may induce a conformational change in the RPS2-RIN4 complex [24].

The relationship between sugars such as glucose and innate plant immunities remain poorly understood. In this study, the effects of glucose on plant immunity in the presence and absence of HXK1 were assessed, and links to PTI and ETI mechanisms were elucidated.

**Materials and methods**

**Plant lines and growth conditions**

*Arabidopsis thaliana* accessions Columbia (Col-0) and Landsberg erecta (Ler) and HXK1 deficient mutant *hxk1* (SALK _034233) (in both the Col-0 and Ler backgrounds) were a kind gift from professor Woe Yeon Kim at the Division of Applied Life Science, Gyeongsang National University, Republic of Korea. Plants were cultivated in a growth chamber with a 16 h light/8 h dark light cycle, light intensity 75 µmol m⁻² s⁻¹, humidity 85±1%, and temperature 22±1 °C. After 2 weeks, seedlings were transferred to a large tray and cultivated until seedlings were 4–5 weeks old. All seedlings were grown following long days (16 h light/8 h dark) to determine the protein accumulation and gene expression levels.

**Bacterial strains and treatment**

*Pseudomonas syringae pv. tomato* DC3000 containing an empty vector plasmid pVSP61(*Pto*), type III effector protein-expressing strains *Pto AvrRpm1* and *Pto AvrRpt2*, *Pto* TTSS-deficient mutant *hrcC−*, and *P. syringae pv. Phaseolicola* (*Pphi*) were provided by Professor David Mackey’s Lab, Ohio State University U.S.A. Bacterial strains were grown at 27 °C for 2 days in King’s broth medium containing appropriate antibiotics. WT and *hxk1* plants were evenly assigned into either the Mock group infiltrate with water 24 h before inoculating with 10 mM MgSO₄, the Mock + *bacteria* (*Pto, P. syringae pv. Phaseolicola* (*Pphi*) or *Pto AvrRpm1* and *Pto AvrRpt2*) infiltrate with water 24 h before inoculating bacteria, or the glu + bacteria group inoculated with 2.5% glucose 24 h before inoculating with bacteria. Growth and symptom analysis of *Pto* DC3000 were conducted as described in [25]. Bacterial solutions were syringe inoculated into 4 to 5 weeks old leaves. Leaf, discs were ground to homogeneity in 10 mM MgCl₂ for all growth experiments, and the titer determined by serial dilution and plating.

**Callose staining**

Four-week-old leaves were syringe-infiltrated with 100 µM flg22 and distilled water or pretreated with water or glucose 24 h before inoculating with flg22 or water as a control and collected after 16 h and stained with methyl blue followed [25], and mounted in 50% glycerol, and examined by fluorescence microscopy (OPTICA, Ponteranica BG Italy). Representative views of these pictures were randomized.

**Western analysis**

Western blot was executed as previously mentioned with slight modification [26]. Approximately 100 µg plant tissue was extracted by mixing with 100 µl protein extraction buffer (100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM DTT, and plant protease inhibitor cocktail) and centrifuging at 13,000 rpm for 10 min at 4 °C. Proteins were quantified by bicinchoninic acid assay. Proteins were separated on a 12% SDS-PAGE gel (Mini-protein, Bio-Rad) and transferred to polyvinylidenefluoride membrane. Anti-PR-1 sera were used at a 1:2000 dilution. Chemiluminescent detection and band quantification were performed using a ChemiDoc XRS system (Bio-Rad).
**RNA analysis**

Total RNA was extracted from approximately 100 µg plant tissue using an RNA extraction kit (QIAGEN), after which RNA was treated with DNaseI. RNA was quantified using a Nanodrop spectrophotometer (DeNovix). Total RNA (approximately 1 µg) was used for cDNA synthesis (ENZYMOMICS). Real-time PCR was performed using a Bio-Rad Real-Time PCR detection system with SYBR Green Super Mix (Cosmo Genetech). Ribosomal protein was used as a control. The following primers were used: FRK1, TCAGAGATCGCTTTGCTTGTA and CTGTAAGCATTTTCCGTCGAGTTC; WRKY29, AAGGATCTCCATACCCGAAGA and TTATGATGAAATTCTCCGGG; Ribosomal protein, CGGACAAATTGAGATTCGTG and ACC ACCACGGAGATCTCCGG. Three biological and two technical replications were conducted.

**Ion leakage and HR assay**

For the ion leakage measurement as previously described [18], 12 leaf discs were collected immediately after inoculation with 2 × 10⁸ CFU/ml bacterial strains *Pso* expressing *AvrRpm1* and *AvrRpt2* and washed with 50 ml of ddH₂O, after 30 min leaf discs were re-suspended into 10 ml ddH₂O. Ion leakage was assessed at different time points. For HR assay WT and *hxk1* plants were pretreated with water as a control and 2.5% glucose 24 h before inoculating with 2 × 10⁸ CFU/ml bacterial strains *Pso* expressing *AvrRpm1* and *AvrRpt2* and 10 mM MgCl₂ as control.

**Results**

**HXK1 positively enhances plant defenses against pathogen infection**

To investigate the role of HXK1 in PTI and ETI, the plant immunity-related functions of HXK1 were evaluated using HXK1 deficient mutants (*hxk1*) generated in the Arabidopsis Col-0 and Ler backgrounds. Five-week-old plants were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*). Typical disease symptoms appeared in inoculated leaves 4 days after inoculation [27]. Both *hxk1* mutants exhibited more severe disease symptoms than the corresponding wild-type (WT) plants (Fig. 1a, c). Bacterial populations in the infected *hxk1* mutants were higher than in WT, consistent with the observed infection phenotype (Fig. 1b, d). These results indicate that HXK1 might play an important role in plant defense, and that absence of HXK1 might negatively affect plant immunity to bacteria, resulting in more severe disease symptoms.

**HXK1 plays a central role in PAMP-triggered immunity**

Previous research showed that expression of FRK1 and WRKY29, two PAMP-response marker genes, was induced by flg22 [28]. To investigate the role of HXK1 in PTI, thus, we found that flg22 was treated in *hxk1* mutant plants and wild type. Lower amounts of FRK1 and WRKY29 transcripts accumulated in a *hxk1* mutant line than in WT (Fig. 2a, b). These results suggest that HXK1 is required for flg22-induced gene expression. Similarly, when *hxk1* mutant plants were infected with the non-pathogenic *P. syringae hrcC* mutant, which lacked a functional type III secretion apparatus, bacterial accumulation was higher in *hxk1* mutant plants compared to WT (Fig. 2c), indicating a positive role for HXK1 in PTI. Callose formation in plants is a defensive response that involves the deposition of polysaccharides to reduce the number of microorganisms that can enter the plant cell [29, 30]. Flg22-induced callose deposition was significantly reduced in *hxk1* mutants compared to WT (Fig. 2d).

PR1 can be used as a marker for PTI due to its role in blocking β-1,3glucanase accumulation [31]. PR1 is expressed by *P. syringae pv. phaseolicola* (*Pto Pph*), a non-host pathogen [32]. PR1 expression by *Pph* was reduced in *hxk1* mutants compared to Col-0 (Fig. 2e), suggesting that the absence of HXK1 reduced susceptibility to bacteria in *Arabidopsis* through reductions in callose deposition and PR1 accumulation.

**HXK1 partly enhances plant immunity through effector-triggered immunity**

Two different effectors were used to assess the role of HXK1 in effector-triggered immunity: avirulent *Psingae* strains *Pto AvrRpm1* and *Pto AvrRpm2*. RPM1 is a resistance protein that recognizes phosphorylated RIN4, leading to the hypersensitive response. RIN4 becomes phosphorylated in response to infiltration of *Pto AvrRpm1*. Infiltration of *Pto AvrRpm2* leads to degradation of RIN4, which is recognized by another plant resistance protein, RPS2, leading to HR [26, 33].

In bacterial growth assays, *Pto AvrRpm1* proliferation was higher in the *hxk1* mutant than in WT (Fig. 3a), suggesting that a lack of HXK1 impaired the response to bacterial infection in *Arabidopsis*. However, no significant differences were found in *Pto AvrRpt2* proliferation between *hxk1* and WT (Additional file 1: Fig. S1a). Inoculation with *Pto AvrRpm1* stimulated HR in WT at approximately 5 h-post-infection (hpi), but this response was delayed in the *hxk1* mutant (Fig. 3b). HR was observed at approximately 9 hpi in both *hxk1* and WT leaves inoculated with *Pto AvrRpt2* (Additional file 1: Fig. S1b). Ion leakage from plant cells is characteristic of
HR-mediated cell death, and an ion leakage assay was therefore used to assess cell death. A faster, more severe cell death response reduces the numbers of bacteria that can spread to neighboring plants tissues. As expected from the results of the HR assay, ion leakage was reduced in *hxk1* compared with WT after *Pto AvrRpm1* infiltration (Fig. 3c), supporting the hypothesis that HXK1 is important for a rapid ETI response to occur. Taken together, our results indicate that HXK1 positively enhances plant defense against *P. syringae* pv. tomato DC3000 infection through its involvement in PTI and its partial involvement in ETI.

**Exogenous glucose positively enhances plant immunity**

Sugars have multiple functions in plants. As well as its involvement in carbohydrate biosynthesis during photosynthesis and its role in respiration, previous research showed that low concentrations of glucose positively regulated root growth and development in *Arabidopsis* seedlings [34]. To investigate the role of glucose in plant immunity, different concentrations of glucose was infiltrated into the leaves of mature 5-week-old *Arabidopsis* plants. Shortly after infiltration, plants showed an HR-like reaction due to the osmotic pressure resulting from the addition of glucose. At lower glucose concentrations, the plants assimilated the exogenous glucose over the following 24 h, releasing the osmotic stress and allowing leaves to return to a normal non-HR state. High concentrations of glucose (5% and above) caused permanent damage to plants (Additional file 1: Fig. S2a). Tolerance to glucose was higher in mature plants than in seedlings, where infiltration with 1.5% or 3% glucose was sufficient to suppress seedling growth (Additional file 1: Fig. S2b) [2]. The direct damage caused to cells as a result of the osmotic stress imposed by high concentrations of glucose was irreversible.

Plant defense is not a unilateral process, but involves bilateral interactions between plants and pathogens. To
investigate the effects of glucose on bacteria, Pto, Pto AvrRpm1, and Pto AvrRpt2 were cultivated in liquid KB medium containing a range of glucose concentrations. Bacterial populations were assessed at various time points after exogenous glucose treatment, and the bacterial growth was impacted at approximately 16 h after treatment (Additional file 1: Fig. S3). After confirming that exogenous glucose suppressed bacterial growth in vitro, similar experiments were performed to understand the effects of glucose on plant growth.

To assess the effects of bacteria on plants in the presence of glucose, low concentrations of glucose were pre-infiltrated into plants leaves followed by infiltration with Pto after 1 day. Disease symptoms (Fig. 4a) and bacterial growth (Fig. 4b) were reduced in the presence of glucose. These results suggest that plant immunity was enhanced by glucose, and that pre-infiltration with glucose may prime the immune response by activating pathways that allow the plant to react more rapidly and more vigorously to pathogen challenge. However, the glucose benefit was lost in the hxx1 mutant both with respect to disease symptoms (Fig. 4c) and bacterial growth (Fig. 4d). This suggests that the role of glucose in plant immunity is linked to the HXK1 pathway, but the specific pathways involved remain unknown.

Glucose positively regulates PAMP-triggered immunity via the HXK1 pathway

Callose deposition was examined in infiltrated WT leaves to assess the effects of glucose on PTI (Fig. 5a). Glucose induced higher levels of callose deposition and production of thicker plant cell walls compared to untreated controls. Thinner plant cell walls are more conducive to bacterial entry. Additional callose deposition was not observed in the hxx1 mutant after treatment with exogenous glucose (Fig. 5b). PR1 accumulation is indicative of the speed and intensity of the PTI response. PR1 accumulation was increased and was more rapid in glucose-treated WT plants than in control plants (Fig. 5c), but no similar effect was seen in the hxx1 mutant (Fig. 5d). Taken together, these results indicate that glucose positively regulates PTI via a HXK1-related pathway.

HXK1 plays a key role in glucose up-regulation of effector-triggered immunity

Effector proteins from Pto AvrRpm1 and Pto AvrRpt2 were used to assess the effects of glucose on ETI. As with PTI, pre-infiltration of glucose prior to bacterial infiltration successfully reduced bacterial growth in WT Arabidopsis exposed to Pto AvrRpm1 or Pto AvrRpt2 (Fig. 6a), but no reductions in bacterial growth or disease
symptoms were seen in the *hxk1* mutant (Fig. 6b). This suggests that glucose activates RIN4-related pathways that enhance plant immunity through ETI. A hypersensitive response assay was performed to further elucidate the function of glucose in ETI. In Col-0, HR was observed soon after *Pto AvrRpm1* and *Pto AvrRpt2* infiltration following glucose pre-infiltration (Fig. 6c). However, glucose did not induce a faster HR response in the *hxk1* mutant (Fig. 6d). Rapid HR limits the number of bacteria that can spread to other parts of the plant and, through the induction of early localized cell death, glucose positively regulates ETI by HKX1-related pathways. Overall, glucose enhanced PTI and ETI after exposure to *P. syringae* pv. tomato DC3000 infection, and this effect was likely mediated by HKX1-related pathways.

**Discussion**

Exogenous sugar supplementation was previously shown to impact bacterial growth [35]. In this study, glucose supplementation suppressed bacterial growth in vitro in a largely dose-dependent manner. Additional file 1: Fig. S3, consistent with previous research [36]. Low concentrations of glucose can enter bacterial cells and activate specific pathways that stop or decrease bacterial growth. High glucose concentrations may inhibit bacterial growth through osmotic pressure. Here, when exogenous glucose was added to bacterial growth media, the glucose concentration changed over time. As glucose concentrations within bacterial cells increased, the osmotic pressure within bacterial cells reduced with respect to the outside environment. Under these circumstances, glucose can be transported by carrier proteins and can then activate cellular pathways to slow bacterial growth. However, when the concentration of glucose outside the bacterial cells is too high, the bacteria experience osmotic stress. This results in dehydration, which also suppresses bacterial growth. It is possible that suppression of bacterial growth directly activates related glucose pathways in the absence of osmotic pressure. Additional research is
required to further elucidate the effects of glucose on bacterial growth.

As well as direct inhibition of bacterial growth by glucose (Additional file 1: Fig. S3), glucose affects bacteria via stimulation of plant responses via HKX1-related pathways. Leaf infiltration with glucose prior to bacterial exposure reduced disease symptoms and bacterial growth in WT Arabidopsis (Fig. 4). Initially, this effect was attributed to direct inhibition of bacterial growth by glucose. However, glucose induced callose deposition and earlier PR1 accumulation in plants (Fig. 5), indicating that additional pathways may be involved in the reduced disease phenotype. The impact of glucose exposure prior to infection was investigated with respect to growth of Pto AvrRpm1 and Pto AvrRpt2 and in a hypersensitive response assay (Fig. 6). The enhanced disease resistance observed in WT plants pre-infiltrated with glucose was not apparent in hxk1 mutant plants, further indicating that this phenomenon was not due primarily to the direct effects of glucose on bacterial cells. Taken together, these results indicate that glucose may activate HKX1-related pathways involved in mediating plant immune responses.

Based on our observations, we propose that HKX1 may be involved in several events simulated by glucose during the ETI response. The hxk1 mutant would be expected to block the effect of glucose in ETI, because bacterial growth was similar with and without glucose upon Pto AvrRpt2 infiltration (Fig. 4b). However, faster HR also

Fig. 4 Exogenous glucose positively enhances plant immunity against Pto. a, c Disease symptoms in Col-0 and hxk1 leaves infiltrated with H2O or 2.5% glucose, followed 1 day later by infiltration with Pto (2 x 10^6 CFU/ml) or MgCl2 (control). b, d Bacterial growth in leaves infiltrated as described in (a, c). Error bars represent the standard error of the mean (n = 3). The asterisk indicates a statistically significant difference (P < 0.05, Student’s t-test). All experiments were conducted three times.
appeared in *hxk1* pre-infiltrated with glucose (Fig. 6d). *Pto AvrRpt2* appears to be more glucose sensitive than *Pto AvrRpm1* (Fig. 6a) which may explain why pre-infiltration still has an effect on HR in the *hxk1* mutant.

Our results indicate that HXK1 plays an important role in mediating the effects of glucose on plant immunity. HXK1 is active in plant immunity in both PTI and ETI, and also serves as a kinase that can phosphorylate glucose to glucose-6-phosphate. Absence of HXK1 in the *hxk1* mutant background led to higher bacterial growth, reduced callose deposition, and a delay in PR1 accumulation during PTI.

Glucose is ubiquitous in plant cells and is a good candidate as a signaling intermediary in plant immune responses. This study investigated the effects of glucose on plant defences. Exogenous glucose successfully reduced bacterial growth and disease symptoms. Low concentrations of exogenous glucose positively enhanced plant immunity via the PTI and ETI mechanisms. Glucose enhanced callose deposition, stimulated gene expression, and led to earlier PR1 accumulation, indicating involvement in the PTI pathway. Glucose also induced earlier cell death to prevent bacterial growth and earlier HR was observed. The effects of glucose on WT immune responses to infection were not observed in a *hxk1* mutant, indicating that exogenous glucose plays a role in plant immunity through HXK1-mediated pathways.

![Fig. 5 Exogenous glucose positively enhances PTI.](image)

**Fig. 5** Exogenous glucose positively enhances PTI. a Callose deposition in Col-0 leaves infiltrated with water or glucose (glc) 24 h before inoculating with 100 μM flg22. b Callose deposition in *hxk1* leaves as described in (a). c PR1 accumulation in Col-0 leaves infiltrated with glucose. d PR1 accumulation in *hxk1* leaves infiltrated with glucose. Coomassie Brilliant Blue (CBB) staining was used to ensure equivalent loading. Experiments were repeated three times.

![Fig. 6 Exogenous glucose positively enhances ETI.](image)

**Fig. 6** Exogenous glucose positively enhances ETI. a Bacterial growth in Col-0 plants exposed to *Pto AvrRpm1* or *Pto AvrRpt2* after pre-infiltration with glucose. b Bacterial growth in *hxk1* plants treated as described in a. c HR in Col-0 leaves exposed to *Pto AvrRpm1*, *Pto AvrRpt2*, or MgCl₂ (control) after pre-infiltration with glucose or H₂O. d HR in *hxk1* leaves treated as described in c. Error bars represent the standard error of the mean (n = 3). The asterisk indicates a statistically significant difference (P < 0.05, Student’s t-test). Similar results were obtained from three independent experiments.
Additional file 1: Figure S1. HXK1 is not involved in RPT2-dependent suppression of Pto AvrRpt2. A. Bacterial accumulation in Col-0 and hxk1 after inoculation with 2 × 10^7 CFU/ml of Pto AvrRpt2. Error bars represent the standard error of the mean (n = 3). B. Ion leakage assay in hxk1 and WT leaves infiltrated with Pto AvrRpt2 or MgCl₂ (control). C. Speed of HR in hxk1 and WT infiltrated with Pto AvrRpt2 or MgCl₂ (control). Experiments were repeated three times with similar results. Figure S2. High concentrations of glucose damaged plants. A. Mature leaves of Col-0 and Ler plants infiltrated with 0, 2.5, 5, 7.5, and 10% glucose (glc) B. Arabidopsis Col-0 and Ler seedlings were grown at MS media containing 0%, 1.5% and 3% glucose. Experiments were conducted three times. Figure S3. Exogenous glucose suppressed bacterial growth in vitro. Growth of P. syringae strains Pto, Pto AvrRpm1, and Pto AvrRpt2 in KB liquid media supplemented with different concentrations of glucose (glc) and 2 × 10^9 CFU/ml of bacteria. Bacterial growth was measured at approximately 16 h after treatment.
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