Glucose-6-phosphate induced changed of stomatal aperture in an irradiance dependent manner

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Abstract. Hexokinase catalyses hexose phosphorylation, which is the key step of sucrose metabolism. In this study, stomatal apertures of Arabidopsis epidermal peel were detected with or without exogenous application of mannose, fructose, glucose, and glucose-6-phosphate (G-6P). The results here showed that G-6P, but not glucose itself, induces stomatal closure in Arabidopsis. Furthermore, detection of stomatal apertures of Arabidopsis hexokinase loss of function with exogenous application of glucose showed that glucose induced stomatal closure was not due to osmotic pressure and it triggered guard cell ROS production depend on hexokinase activity. The effect of irradiance and G-6P on regulation of Arabidopsis stomatal aperture was investigated. The data obtained here indicated that G-6P induced changes of stomatal aperture depend on irradiance.

1. Introduction
Higher plants synthesize sugars in leaves by photosynthesis and transport them primarily in the form of sucrose through the phloem to different sink tissues. Sucrose breakdown and glycolysis within guard cells plays an important role in light-induced stomatal opening [1]. Sucrose decomposition is catalysed mainly by invertase or sucrose synthase. The activity of vacuolar invertase was positively correlated with stomatal aperture [2]. Furthermore, based on the optimum pH of invertase and the linkage between the pH in guard cell vacuoles and stomatal aperture [3][4], it was suggested that vacuolar invertase regulates stomatal behaviour in a pH dependent manner [5]. In addition, overexpression of sucrose synthase gene in guard cells promoted stomatal opening [6], while silencing of the sucrose synthase gene by antisense RNA resulted in stomatal closure [7]. After sucrose breakdown, glucose and fructose could be phosphorylated by hexokinase for glycolysis and other metabolic pathways. The first plant hexokinase was identified from Arabidopsis thaliana by functional complementary expression in yeast [8]. Thereafter, hexokinase genes have been cloned from many plant species, such as maize, pear, potato, rice, sunflower, tomato, Jatropha curcas [9][10][11][12][13] [14][15][16]. However not all members have the catalytic function of hexose phosphorylation. For instance, among the six genes from Arabidopsis, only three of them encoded a protein with hexose phosphorylation activity [17]. Arabidopsis HXK1 (AtHXK1) has been shown to be hexokinase gene family member with hexose-phosphorylation activity [18]. Overexpression of HXK1 resulted in stomatal closure in tomato, citrus and Arabidopsis [19][20][21]. However, it remains unknown whether hexokinase or the product of the catalysed reaction, hexose-phosphate, that exerts the regulation on stomatal function.
In addition, transpiration rates of transgenic citrus plants overexpressing AtHXK1 in guard cells remains constant under different irradiance while transpiration rates of wild-type citrus plants peaked and then declined as the intensity of the light increased [21]. Thus, it is worth to investigate how stomatal behaviour affected by sugars under different irradiance. Exogenous sucrose and glucose application lead to stomatal closure of detached leaves incubated in stomatal opening buffer [1][22]. To investigate the possible mechanism for exogenous sugar-induced stomatal closure, the production of reactive oxygen species (ROS) from guard cell was measured by fluorescent probe, which revealed that glucose- and ABA- induced stomatal closure was mediated by ROS production in guard cells [22][23]. Indeed, a link between ROS production in guard cells and stomatal closure was demonstrated by metabolic profile of guard cells in response to high level of CO2 [24]. However, it is still unclear how glucose triggers ROS production.

In this paper, we report that low hexokinase activity altered stomatal density and exogenous glucose-6-phosphate (G-6P) resulted in changes of stomatal aperture of detached leaf peels. We also use Arabidopsis T-DNA insertion mutant lines with low hexokinase activity to show that G-6P or glucose phosphorylation, but not glucose itself, induced stomatal closure and triggered ROS production in guard cells of detached leaf peels.

2. Materials and methods

2.1. Plant materials
Arabidopsis thaliana T-DNA insertion lines (CS69153, CS864200) were obtained from the Arabidopsis biological resource center. Seeds of Arabidopsis wild type (Col-0) and aforementioned T-DNA insertion mutant lines were surface sterilized followed by transplantation on soil and grown in green house according to our previous study [5].

2.2. Measurement of stomatal aperture and density
To measure the stomatal aperture, abaxial epidermal peels of 4 week old Arabidopsis fully expanded leaves were incubated in a stomatal opening buffer with 10 mM potassium chloride, 7.5 mM iminodiacetic acid, 10 mM MES (pH=6.15) for 2 hours under dark followed by 2 hours under light according to the protocol described by Jin et al [25]. The treated peels were observed by using a light microscopy (Motic BA300). The abaxial stomata images were photographed and the stomatal aperture (ratio of stoma width to length) was calculated from more than 50 stoma pores from 5 - 6 peels derived from 3 leaves using image software (Image J). For stomatal density measurement, more than 30 pictures of microscopic field with abaxial stomata images were calculated.

2.3. Drought stress treatment
Fresh weight (FW) loss of detached leaves of 4-week-old wild-type and T-DNA insertion lines was measured at different time point. Relative water content was measured according to our previous study [5].

2.4. Hexokinase activity analysis
Hexokinase activity was measured according to the protocol described by Dai et al.[26] and it was calculated as quantity of G-6P formed by glucose phosphorylation per hour and per mg total protein.

2.5. Measurement of reactive oxygen species production in guard cells
Reactive oxygen species (ROS) production in guard cells was measure by following the protocol described by Li et al [22] with some modifications. The epidermal peels were incubated in the stomatal opening buffer as aforementioned for 2 hours under dark followed by adding 100 mmol of glucose and incubated for 2 hours under light. And then ROS (reactive oxygen species) fluorescence probe (2',7'-dichlorofluorescin di-acetate) was added and incubated under dark condition for 15 min. After washing
excess probe, the fluorescence signal was observed with a microscope, and ROS production was calculated with fluorescent intensity by Image J.

2.6. Statistical analysis
All experiments here were repeated more than three times with three biological replicates each time. One leaf was sampled from one plant for each biological replicate. Standard deviations were used to assess the differences between samples.

3. Results
It was reported that exogenous hexoses induced stomatal closure in *V. faba* [22]. Since hexose could be phosphorylated to hexose phosphate, it prompts us to test whether it was hexoses themselves or its phosphorylated form induce stomatal closure in *Arabidopsis*. The abaxial epidermal peels of *Arabidopsis* wild-type plants were treated with exogenous hexoses (glucose, mannose, fructose) and phosphorylated form of glucose (G-6P). Similar to the results acquired from *V. faba* [22], the three hexoses led to stomatal closure in *Arabidopsis* as expected (Fig. 1). In addition, we found that phosphorylated form of glucose, G-6P also led to stomatal closure in *Arabidopsis* (Fig. 1).

![Figure 1](image_url)

**Figure 1** Mannose-, fructose-, glucose-, and G-6P induced stomatal closure in epidermal peels of *Arabidopsis*. The concentration of mannose-, fructose-, glucose-, and G-6P was 100 mM.

Irradiance response pattern of transpiration rates of transgenic citrus plants overexpressing *AtHXK1* is different from that of wild-type plant [21]. To investigate if G-6P, the phosphorylation form of glucose, also affect Arabidopsis stomatal aperture response pattern to irradiance, we measured stomatal aperture of *Arabidopsis* epidermal peels treated with or without exogenous glucose and G-6P under different irradiance. The epidermal peels were incubated in the stomatal opening buffer as aforementioned for 2 hours under dark followed by exposure to different irradiance for 2 hours. Stomatal aperture of epidermal peels increased with the increase of irradiance when it was less than 700μmol·m⁻²·s⁻¹, but decrease when it was more than 700μmol·m⁻²·s⁻¹. On the other hand, stomatal aperture of epidermal peels treated with G-6P increased with the increase of irradiance even it was more than 700μmol·m⁻²·s⁻¹. Moreover, stomatal aperture of epidermal peels treated with G-6P was less than that without G-6P treatment under dark or when the irradiance was not so high, and contrary results was observed under high l irradiance (Fig 2). This result implied that G-6P induced changed of stomatal aperture depend on irradiance.
Figure 2 G-6P induced changes of stomatal aperture depend on irradiance.

Reactive oxygen species (ROS) production in guard cells was demonstrated to mediate stomatal closure in V. faba induced by hexoses [22]. To further understand the possible mechanism for ROS production and stomatal regulation, Arabidopsis hexokinase loss-of-function mutants were used to examine glucose-triggered ROS production. Like the results from V. faba, more ROS was produced in guard cells of epidermal peels of wild-type plants treated with glucose than that untreated control, indicating that glucose also triggered ROS production in guard cell of epidermal peels of Arabidopsis leaves. However, ROS production in guard cells of epidermal peels of hexokinase loss-of-function mutant plants treated with glucose was more or less the same as the untreated control, suggesting that glucose did not trigger guard cell ROS production in epidermal peels of the mutant lines (Fig 3). To confirm if this phenomenon is directly induced by mutation of hexokinase gene, we measured hexokinase activity in wild-type plants and the mutant lines. The two hexokinase loss-of-function mutant lines displayed a significantly lower hexokinase activity compared to wild-type plants (Fig 4a). All these results suggested that G-6P or glucose phosphorylation, but not glucose itself, induced stomatal closure and trigger ROS production.

Figure 3 Glucose induced stomatal closure triggered guard cell ROS production depend on hexokinase activity. (a) glucose induced stomatal aperture in Arabidopsis wild-type plants, but not in hexokinase loss-of-function mutants. (b) Glucose triggered guard cell ROS production in Arabidopsis wild-type plants, but not in hexokinase loss-of-function mutants. hxl-2 and hxl-1 were hexokinase loss-of-function mutant lines. Asterisks indicate significant differences (Student’s t-test, *p<0.05 and **p<0.01) between the mutant plants and the wild-type plants.
Figure 4 Lose of hexokinase activity increased stomatal density. (a) Hexokinase activity of hexokinase loss-of-function mutants was less than that of Arabidopsis wild-type plants. (b) Stomatal density of hexokinase loss-of-function mutants was more than that of Arabidopsis wild-type plants. hxk1-2 and hxk1 were hexokinase loss-of-function mutant lines. Asterisks indicate significant differences (Student’s t-test, *p<0.05 and **p<0.01) between the mutant plants and the wild-type plants.

The studies on tomato and Arabidopsis hexokinase over-expression and loss-of-function mutants revealed that hexokinase activity is negatively correlated to stomatal aperture and stomatal conductance [20]. To further understand the role of Arabidopsis hexokinase gene, we measured stomatal density of hexokinase loss-of-function mutant plants. The analysis revealed higher stomatal density of hexokinase loss-of-function mutant lines than that of wild-type plants (Fig 4b). Therefore, we compared the fresh weight loss of detach leaves and relative water content between wild-type plants and hexokinase loss-of-function mutant lines. Fresh weight in detach leave of hexokinase loss-of-function mutant lines was much less than in those of wild-type plants, indicating the mutant lines loss more water than that in wild-type plants per unit time (Figure 5). These results indicated that loss of hexokinase activity in Arabidopsis increase stomatal density and thus display sensitive to drought stress.

Figure 5 Lose of hexokinase activity leads to drought sensitivity in Arabidopsis.
Fresh weight loss was significantly higher in Arabidopsis hexokinase loss-of function mutants than that of wild-type plants. hxk1-2 and hxk1 were hexokinase loss-of-function mutant lines. Asterisks indicate significant differences (Student’s t-test, *p<0.05 and **p<0.01) between the mutant plants and the wild-type plants.

4. Discussion
Here, we show that both of exogenous glucose and G-6P application reduced stomatal aperture of wild-type plants. However, application of exogenous glucose did not alter stomatal aperture of epidermal peels of hexokinase loss-of-function mutant plants. These results indicated that G-6P, the
phosphorylation form of glucose, but not glucose itself, induce stomatal closure. Support to this conclusion also comes from observations that hexokinase activity or its expression level is negatively correlated with stomatal aperture [20][21]. Hexokinase was regard as glucose sensor in guard cells [20][27][28][29][30]. However, the exact mechanism by which hexokinase regulates stomatal movement remains to unclear. Our data suggested that stomatal movement is regulated by G-6P or even the downstream products of glucose metabolism. We also used Arabidopsis mutants with low hexokinase activity to show that glucose triggers ROS production and induces stomatal closure based on phosphorylation.

It was reported that stomatal conductance and transpiration rate of Arabidopsis mutants was bigger than wild-type plants [20]. Accordingly, we found that the fresh weight loss was significantly higher in hxk1-2 and hxk1-3, Arabidopsis hexokinase loss-of function mutants, than that of wild-type plants. The increase of stomatal conductance and water loss rate of detached leaves in hxk1-2 and hxk1-3 could be due to changes of stomatal aperture and/or density compared to wild-type plants. Our results here indicated that there is no significant difference between stomatal aperture of Arabidopsis hexokinase loss-of function mutants and that of wild-type plants. Therefore, higher stomatal conductance and fresh weight loss in Arabidopsis hexokinase loss-of function mutants should be due to increase of stomatal density observed here. Stomatal density regulated by lots of factors. For instance, TMM (too many mouth) is the earliest identified Arabidopsis stomatal development regulation gene, and tmm-1 mutant displayed increased stomatal density [31]. Mitogen-activated protein kinase, MPK3 and MPK6 is the negative factor of stomatal development and patterning, and epidermal of cotyledon of mpk3-/- mpk6-/- double mutant made up of clusters of stoma [32]. Loss of function mutant of transcription factor SPEECHLESS (sph-1) lack stoma at all [33]. Stomatal density is also associated with mesophyll conductance[34] and leaf primordium can recognize CO2 concentration and irradiance and thereafter adjust leaf stomatal density[35]. Therefore, we speculate that the low activity of hexokinase in plants affects the photosynthetic efficiency and is recognized by leaf primordium, which results in the increase of stomatal density

Here we also showed that stomatal aperture increased till irradiance reached to 700μmol·m−2·s−1 and decreased when irradiance exceeded 700μmol·m−2·s−1. Meanwhile, stomatal aperture of epidermal peels treated with G-6P increased with the increase of the irradiance even the irradiance has exceeded 700μmol·m−2·s−1. High level of sugars was reported to reduce photosynthesis rate[26], and it might be due to sugar induced stomatal closure. Stomatal aperture of epidermal peels treated with G-6P was observed under high irradiance bigger than that of control might to be due to that high irradiance increases photosynthesis rate, and thus induces stomatal opening and adapts high level of sugars. It was not same as transpiration rate of citrus plants overexpressing AtHXH1 response to different irradiance [21]. However, both overexpression of HXK1 and G6P treatment decreased the response of stomata to irradiance compared to control.

5. Conclusions
From the results here, we can draw following conclusions. G-6P, but not glucose itself, induces stomatal closure in Arabidopsis. Glucose induced stomatal closure, and it triggered guard cell ROS production depend on hexokinase activity. G-6P induced changes of stomatal aperture depend on irradiance.

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