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RESEARCH PAPER

Glucose signalling positively regulates aliphatic glucosinolate biosynthesis

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Abstract

The effects of glucose on aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana* were investigated in this study by using mutants related to aliphatic glucosinolate biosynthesis and regulation, as well as glucose signalling. The results showed that glucose significantly increased the contents of individual and total aliphatic glucosinolates. Expression of *MYB28* and *MYB29*, two key transcription factors in aliphatic glucosinolate biosynthesis, was also induced by glucose. Consistently, the increased accumulation of aliphatic glucosinolates and the up-regulated expression of *CYP79F1* and *CYP79F2* induced by glucose disappeared in the double mutant *myb28myb29*. MYB28 and MYB29 synergistically functioned in the glucose-induced biosynthesis of aliphatic glucosinolates, but MYB28 was predominant over MYB29. Interestingly, the content of total aliphatic glucosinolates and the expression level of *MYB28* and *MYB29* were substantially reduced in the *glucose insensitive* mutant *gin2-1* and the ABA insensitive 5 (*abi5-7*) mutant compared with the wild type. In addition, total aliphatic glucosinolates accumulated much less in another sugar-insensitive RGS1 (regulator of G-protein signaling 1) mutant (*rgs1-2*) than in the wild type. These results suggest that glucose-promoted aliphatic glucosinolate biosynthesis is regulated by HXK1- and/or RGS1-mediated signalling via transcription factors, MYB28, MYB29, and ABI5.

Key words: ABI5, aliphatic glucosinolates, glucose, hexokinase1 (HXK1), regulation, RGS1.

Introduction

Glucosinolates are a group of nitrogen- and sulphur-containing secondary metabolites found mainly in *Brassicaceae* crops. They can be grouped into aliphatic, aromatic, and indolic glucosinolates based on their different side chain structures (Grubb and Abel, 2006; Agerbirk and Olsen, 2012). It is well known that glucosinolates and their degradation products have diverse biological functions that range from anticarcinogenic activities to plant defence against pathogens and herbivores (Fahey *et al*., 1997; Grubb and Abel, 2006; Yatusevich, 2008; Kos *et al*., 2012; Saavedra *et al*., 2012). Aliphatic glucosinolates have been demonstrated to play an important role in plant–herbivore interactions and non-host resistance in the *Arabidopsis–Pseudomonas* pathosystem (Beekwilder *et al*., 2008; Fan *et al*., 2011).

To date, almost all genes involved in the glucosinolate biosynthetic pathway have been identified (Yan and Chen, 2007; Sønderby *et al*., 2010). *CYP79F1* and *CYP79F2* are two important genes whose products catalyse accumulation of long-chain aliphatic glucosinolates, while the product of *CYP79F1* also functions in the biosynthesis of short-chain

Abbreviations: ABI5, ABA insensitive5; gin2, glucose insensitive2; HXK1, hexokinase1; RGS, regulator of G-protein signalling.

aliphatic glucosinolates (Reintanz *et al*., 2001; Chen *et al*., 2003; Tantikanjana *et al*., 2004). In addition, MYB28, MYB29, and MYB76 have been identified as transcription factors in aliphatic glucosinolate biosynthesis with partial functional redundancy. Among them, MYB28 plays the most important role in aliphatic glucosinolate biosynthesis, followed by MYB29 and MYB76 (Hirai *et al*., 2007; Gigolashvili *et al*., 2009). Furthermore, many abiotic factors including nitrogen and sulphur nutrients as well as the plant hormones have been reported to affect the profile and content of glucosinolates (Kliebenstein *et al*., 2002; Mikkelsen *et al*., 2003; Mewis *et al*., 2005; Aires *et al*., 2006; Chen *et al*., 2006; Falk *et al*., 2007; Bano, 2010; Chen *et al*., 2011; Liu *et al*., 2011).

Sugars play important roles in plant growth and development as a carbon and energy source. They can also act as effective signalling molecules throughout plant life (Rolland *et al*., 2006; Ramon *et al*., 2008; Bolouri-Moghaddam *et al*., 2010; Smeekens *et al*., 2010). Hexokinase (HXK), the first enzyme involved in glucose catabolism, can sense glucose and initiate the signalling pathway in *Arabidopsis* (Moore *et al*., 2003). The network of HXK1-dependent glucose and ABA signalling has been identified, and the ABA-insensitive *abi5* mutant is insensitive to glucose (León and Sheen, 2003; Ramon *et al*., 2008). Furthermore, it is reported that a regulator of G-protein signalling (AtRGS1) acts as a cell surface receptor and functions in a HXK-independent glucose signalling pathway (Chen and Jones, 2004; Rolland *et al*., 2006). In previous studies, sugar-regulated plant secondary metabolites were observed in several *Brassica* vegetables (Guo *et al*., 2011; Wei *et al*., 2011). The induction of anthocyanin biosynthesis by sucrose has been clearly illustrated (Teng *et al*., 2005). Cross-talk between sucrose and hormone signalling pathways in the regulation of the anthocyanin biosynthetic pathway has also been reported (Loreti *et al*., 2008). Apart from the reports showing that glucose induces the expression of *MYB28* in *Arabidopsis* (Li *et al*., 2006; Gigolashvili *et al*., 2007), little information is available on the role of glucose in aliphatic glucosinolate accumulation. In this study, the aim was to investigate the regulatory mechanism of glucose on aliphatic glucosinolate biosynthesis, and to identify the components involved in glucose signalling.

Materials and methods

Plants and growth conditions

Seeds were sterilized for 30 s in 75% ethanol and washed with sterile water twice, and then immersed in 10% sodium hypochlorite for 2 min, followed by washing with sterile water four times. Except for the experiments shown in Supplementary Figs S1, S3, and S6, available at *JXB* online, plant growth conditions in all experiments were as follows. The seeds were stratified for 3 d at 4 °C, and transferred into flasks (~50 seeds per flask) with 40 ml of liquid growth medium [full-strength sterilized Murashige–Skoog (MS) salt solution+0.5% glucose]. Plants were grown under a photoperiod of 16 h light/8 h dark with gentle shaking (135 rpm) for 10 d in a plant growth chamber at 21 °C (Loreti *et al*., 2008). For Supplementary Figs S1 and S3, experiments were carried out with solid culture as follows: surface-sterilized seeds were planted on 0.5% agar plates containing MS salt and 0.5% glucose. In the experiment shown in Supplementary Fig. S6, MS salt solution was replaced with half-strength sterilized MS salt solution. Seeds were grown in a plant growth chamber under the same conditions mentioned above after being incubated at 4 °C for 3 d. Mutant seeds of *gin2-1* were generously provided by Dr Sheng Teng (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences). The *rgs1-2* seeds was obtained from Dr Jirong Huang (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences). Mutant seeds of *myb28* (SALK_136312) and *myb29* (CS121027) were purchased from ABRC (Arabidopsis Biological Resource Center) (Hirai *et al*., 2007; Beekwilder *et al*., 2008). The double knock-out mutant *myb28myb29* was a gift from Dr Piero Morandini, University of Milan, Italy (Beekwilder *et al*., 2008). The genetic background of all mutants was Columbia (Col-0), except for *gin2-1* which was Landsberg (L*er*).

Glucose and sorbitol treatments

Sterilized glucose and sorbitol were added to the flasks after 10 d at final concentrations of 1, 3, and 5% (w/v) with water as a control. For solid culture, 10-day-old seedlings were transferred to MS agar plates with 3% glucose or 3% sorbitol. After treatments, plants were cultured in the same condition as before and were collected at different time points for analysis.

Glucosinolate assay

Seedlings were harvested 1, 3, and 5 d after treatment. Glucosinolates were extracted and analysed as previously described, with minor modifications (Sun *et al*., 2011*a*, *b*). Fresh tissues (100 mg) were boiled in 1 ml of water for 10 min. After recovery of the liquid, the residues were washed with water (1 ml), and the combined aqueous extract was applied to a DEAE-Sephadex A-25 (30 mg) column (pyridine acetate form). The column was washed three times with 20 mM pyridine acetate and twice with water. The glucosinolates were converted into their desulpho analogues by overnight treatment with 100 µl of 0.1% (1.4 U) aryl sulphatase, and the desulphoglucosinolates were eluted with 2×0.5 ml of water. The high-performance liquid chromatography (HPLC) analysis was performed using Shimadzu HPLC (Shimadzu, Kyoto, Japan), consisting of two LC-20AT solvent delivery units, a DGU-20A3 degasser, a CTO-10ASVP column oven, an SIL-20A autosampler, and an SPD-M20A diode array detector. The HPLC system was connected to a computer with LC solution Version 1.25 Software. A Hypersil C18 column (5 µm particle size, 4.6 mm×250 mm; Elite Analytical Instruments Co. Ltd, Dalian, China) was used with a mobile phase of acetonitrile and water at a flow rate of 1.0 ml min^{-1}. The procedure employed isocratic elution with 1.5% acetonitrile for the first 5 min; a linear gradient to 20% acetonitrile over the next 15 min; followed by isocratic elution with 20% acetonitrile for the final 13 min. A 20 µl sample was injected onto the column by an autosampler. Absorbance was detected at 226 nm. Sinigrin (Sigma, St Louis, MO, USA) was used as an internal standard for calculation of molar concentrations of individual glucosinolates, and relative response factors were applied to correct absorbance differences between the standard and other glucosinolates (Brown *et al*., 2003). Data were given as nmol mg^{-1} FW (fresh weight).

RNA isolation and expression analysis

Seedlings were collected at different time points (0, 6, 12, 18, 24, and 36 h after treatment) and immediately immersed in liquid nitrogen. Total RNA was isolated from ~100 mg of *Arabidopsis* leaves using the Trizol reagent according to the manufacturer's instruction (Takara, Japan). RNA samples were reversed transcribed into cDNAs using Prime Script RT Master Mix (Takara, Japan).

Real-time quantitative PCR (qPCR) was performed in a total volume of 20 µl, including 2 µl of diluted cDNA, 1 µl of each primer (5 µM), and 10 µl of 2× SYBR Green PCR Master Mix (Takara, Japan) on an Applied Biosystems StepOne Real-Time PCR Systems

(Applied Biosystems, USA) according to the kit manual. The qPCR program was conducted at 95 °C for 10 s first, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The expression level of *Arabidopsis ACTIN2* was used as an internal control and the expression of other genes was computed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primers used in this work are listed in Table 1.

Statistical analysis

Statistical analysis was performed using the SPSS package program version 11.5 (SPSS Inc., Chicago, IL, USA). For Figs 1–3, and Supplementary Figs S1, S2, and S4–S6 at *JXB* online, differences in glucosinolate accumulation among different treatments were tested, while differences in Fig. 5A were tested among different mutants. They all were analysed by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test at a 95% confidence level ($P < 0.05$). For Figs 6A, B, 7A, and Supplementary Fig. S3, differences in glucosinolate accumulation among different mutants were analysed using independent-samples *t*-test. The values are reported as means with standard error for all results.

Results

Effect of glucose on glucosinolate contents in Arabidopsis

To study the role of glucose in glucosinolate biosynthesis, 10-day-old seedlings of *Arabidopsis* wild-type (Col-0) were treated with different concentrations (1, 3, and 5%) of glucose or sorbitol (as an osmotic control) and sampled 1, 3, and 5 d after each treatment. As shown in Fig. 1, the contents of 4MSOB (4-methylsulphinylbutyl glucosinolate, the predominant aliphatic glucosinolate) and the total aliphatic glucosinolates increased significantly at almost all time points under all the tested concentrations except for 1 d after 1% glucose or sorbitol treatment. Similarly, the contents of I3M (indol-3-ylmethyl glucosinolate, the predominant indolic glucosinolate) and the total indolic glucosinolates were also enhanced significantly by glucose (Fig. 2). Furthermore, glucose promoted glucosinolate accumulation in a time-dependent manner (Figs 1, 2). It should be noted that glucose stimulated accumulation of glucosinolates much more than sorbitol, indicating that the regulatory mechanism of glucose and sorbitol on glucosinolate biosynthesis is different.

Since vitrification occurred in some seedlings 5 d after treatment, the effect of glucose on glucosinolate biosynthesis was studied using plants treated with a moderate concentration (3%) for 3 d. Under this condition, the contents of major aliphatic glucosinolates, 3MSOP (3-methylsulphinylpropyl glucosinolate), 4MTB (4-methylthiobutyl glucosinolate), and 4MSOB, and total aliphatic glucosinolates notably increased by 146, 30, 38, and 31%, respectively, compared with sorbitol treatment (Fig. 3A). In addition, significant increases in 4MOI3M (4-methoxyindol-3-ylmethyl glucosinolate), I3M, and total indolic glucosinolates were also observed 3 d after 3% glucose treatment compared with sorbitol treatment (Fig. 3B).

To verify the effect of glucose on aliphatic glucosinolate biosynthesis, solid media were used to grow seedlings (for details see the Materials and methods). The results showed the same trend as the liquid culture (Supplementary Fig. S1 at *JXB* online). Because there exists an interaction between glucose and nitrogen (Price *et al*., 2004), liquid growth medium including half-strength MS salt solution with 0.5% glucose, which contains a lower nitrogen concentration, was used to confirm the induction of glucose, and strong induction of glucose still can be observed (Supplementary Fig. S6). In addition, the content of total aliphatic glucosinolates was also measured at 0, 6, 12, 24, and 36 h after glucose treatment to investigate the time-course accumulation of aliphatic glucosinolates (Supplementary Fig. S2). A significant difference in total aliphatic glucosinolates between glucose and sorbitol treatment occurred 24 h after treatment.

Glucose induces expression of transcription factors and biosynthetic genes in the aliphatic glucosinolate pathway

MYB28 and *MYB29* are two major regulators for aliphatic glucosinolate biosynthesis, and the double mutant *myb28myb29* does not accumulate any aliphatic glucosinolates (Gigolashvili *et al*., 2007, 2008; Hirai *et al*., 2007; Sønderby *et al*., 2007; Beekwilder *et al*., 2008). *CYP79F1* and *CYP79F2* encode two core biosynthetic enzymes catalysing the production of aliphatic glucosinolates (Tantikanjana *et al*., 2004;

Table 1. Primer sequences used for quantitative real-time polymerase chain reaction (PCR).

Target gene	Locus code	Primer sequence	Reference
ACTIN2	AT3G18780	5'-TAACTCTCCCGCTATGTATGTCGC-3' 5'-CCACTGAGCACAATGTTACCGTAC-3'	Gigolashvili *et al*. (2007)
CYP79F1	AT1G16410	5'-CCATACCCTTTTCACATCCTACTAGTCT-3' 5'-GTAGATTGCCGAGGATGGGC-3'	Gigolashvili *et al*. (2007)
CYP79F2	AT1G16400	5'- ACTAGGATTTATCGTCTTCATCGCA-3' 5'-CTAGGACGAGTCATGATTAGTTCGG-3'	Gigolashvili *et al*. (2007)
MYB28	AT5G61420	5'-TCCCTGACAAATACTCTTGCTGAAT-3' 5'-CATTGTGGTTATCTCCTCCGAATT-3'	Gigolashvili *et al*. (2007)
MYB29	AT5G07690	5'-CAATACTGGAGGAGGATATAACC-3' 5'-AGTTCTTGTCGTCATAATCTTGG-3'	Beekwilder *et al*. (2008)
MYB76	AT5G07700	5'-ACGTTTAATCGATGATGGCA-3' 5'-ATGGGCTCAACTGGATTAGG-3'	Designed by software www.genscript.com

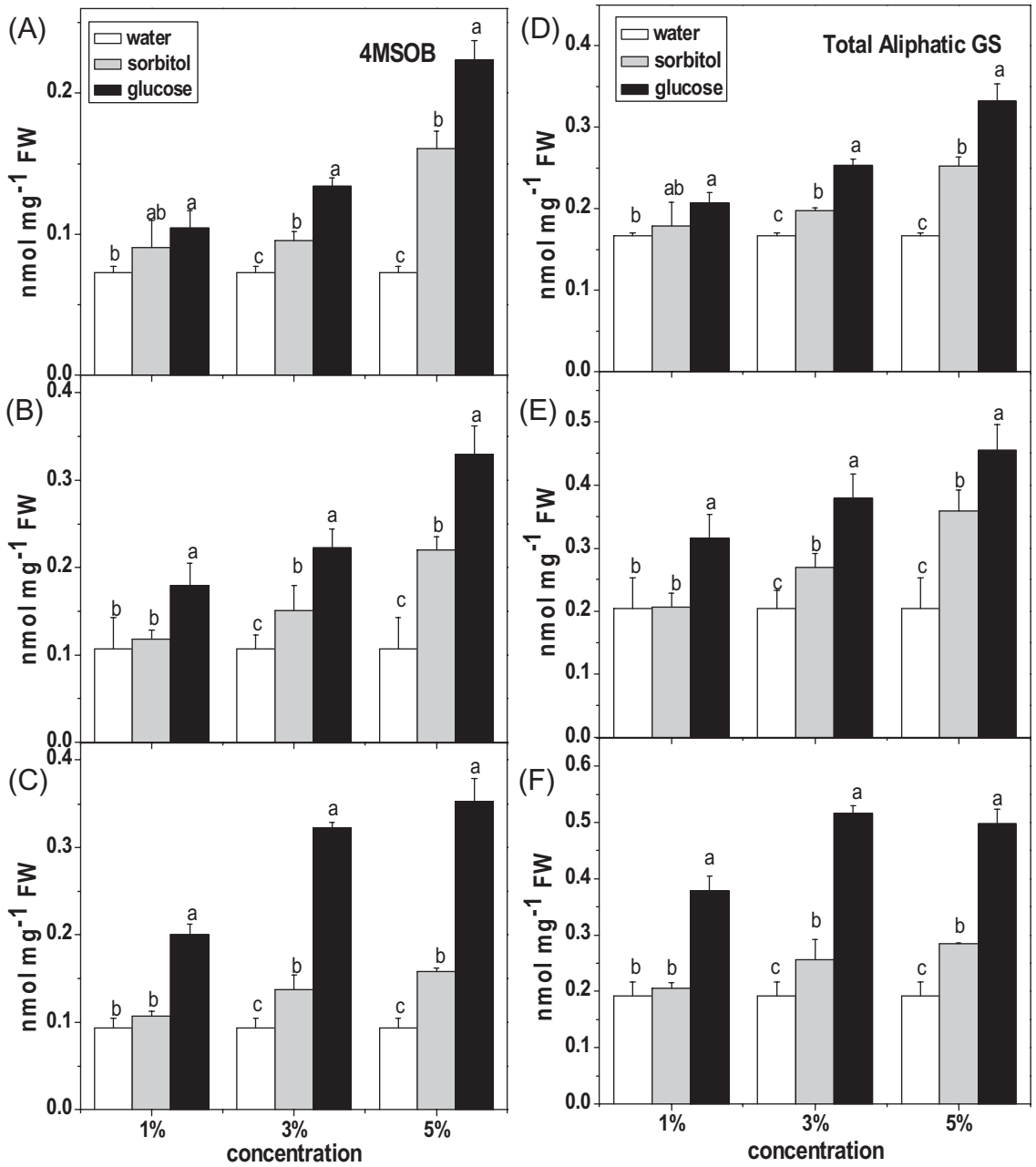

Fig. 1. Effect of glucose on accumulation of aliphatic glucosinolates (GS) in a time-course experiment. Total aliphatic glucosinolates and 4MSOB were measured in 10-day-old *Arabidopsis* seedlings treated with 1, 3, and 5% glucose, and sorbitol. Samples were collected after 1 d (A, D), 3 d (B, E), and 5 d (C, F). Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$.

Sønderby *et al*., 2010). Here the data showed that the expression of all the four genes responded positively to exogenous glucose treatment at a time point as early as 6 h (Fig. 4). The expression of the genes was induced with a steady rise after 6 h, peaked at 18 h (*MYB28* and *CYP79F2*) or 24 h (*MYB29* and *CYP79F1*), and then decreased sharply at 36 h. The expression levels of *MYB28*, *MYB29*, *CYP79F1*, and *CYP79F2* increased by ~8.4-, 2.5-, 1.8-, and 1.3-fold, respectively, at 18 h after the 3% glucose treatment when compared with sorbitol treatment. So, 18 h after treatment was set as the harvest point of *Arabidopsis* plants for further analysis of gene expression.

Effect of glucose on aliphatic glucosinolate biosynthesis in myb28, myb29 and myb28myb29 mutants

The level of total aliphatic glucosinolates was examined in *myb28* and *myb29* under glucose treatment. As shown in Fig. 5A, the level of total aliphatic glucosinolates decreased significantly in both mutants, especially in *myb28*. In *myb28*, although the contents of 4MTB and 4MSOB increased with glucose treatment (data not shown), the content of total aliphatic glucosinolates was much lower than that in the wild

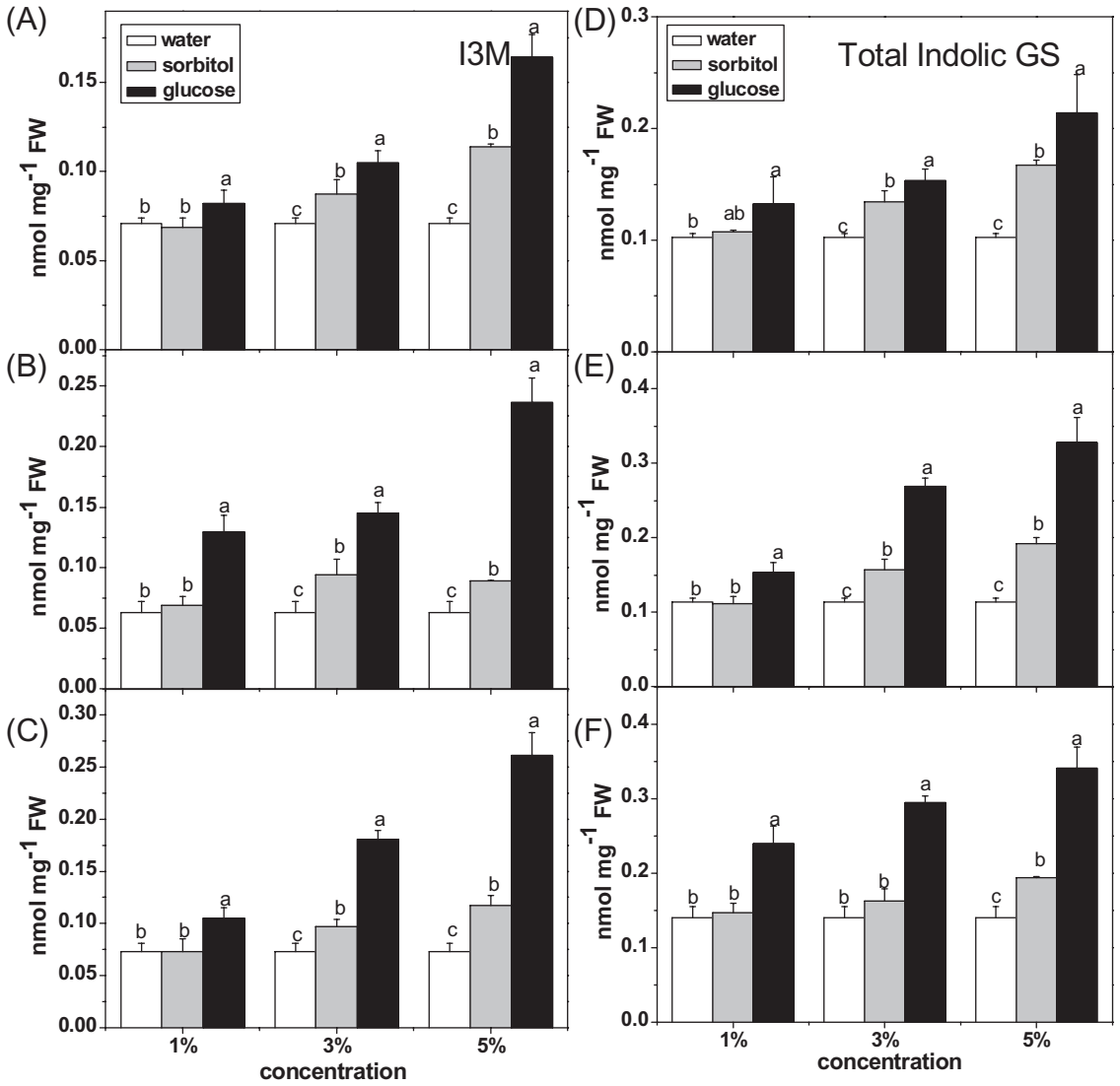

Fig. 2. Effect of glucose on accumulation of indolic glucosinolates in a time-course experiment. Total indolic glucosinolates and I3M were measured in 10-day-old *Arabidopsis* seedlings treated with 1, 3, and 5% glucose, and sorbitol. Samples were collected after 1 d (A, D), 3 d (B, E), and 5 d (C, F). Each data point represents the mean of three independent biological replicates (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$.

type. Interestingly, glucose treatment rescued the chemotype of low contents of individual and total aliphatic glucosinolates in the *myb29* mutant.

The transcriptional level of *MYB28*, *MYB29*, *CYP79F1*, and *CYP79F2* was determined in *myb28* and *myb29* mutants. As shown in Fig. 5B, glucose treatment dramatically increased the expression level of *MYB28* in both wild-type and *myb29* plants compared with sorbitol treatment, which produced a slightly increased *MYB28* expression level. *MYB29* expression was also substantially induced by glucose in the wild type, but glucose-induced expression of *MYB29* was very weak in *myb28* (Fig. 5C). The expression pattern of *CYP79F1* and *CYP79F2* was similar to that of *MYB28* (Fig. 5D, E). These results indicate that *MYB28* is a key transcription factor for expression of genes involved in aliphatic glucosinolate biosynthesis. No detectable aliphatic glucosinolates were found in the *myb28myb29* double mutant with or without glucose treatment (Fig. 5A). Consistently, the expression levels of *CYP79F1* and *CYP79F2* were almost undetectable in the double mutant (Fig. 5F, G).

Effect of glucose on aliphatic glucosinolate biosynthesis in gin2-1 and rgs1-2 mutants

The HXK1 null mutant *gin2-1* is glucose insensitive (Moore *et al*., 2003), while *rgs1-2*, a null mutant of the gene encoding RGS protein (AtRGS1), is insensitive to glucose and sucrose (Chen and Jones, 2004). Accumulation of total aliphatic glucosinolates was analysed in these two mutants along with their wild types under liquid culture. As shown in Fig. 6A and B, the level of total aliphatic glucosinolates in *gin2-1* and *rgs1-2* decreased by 46% and 23%, respectively, when compared with the corresponding wild types. Similar results were also observed when the mutants were grown on the solid culture medium

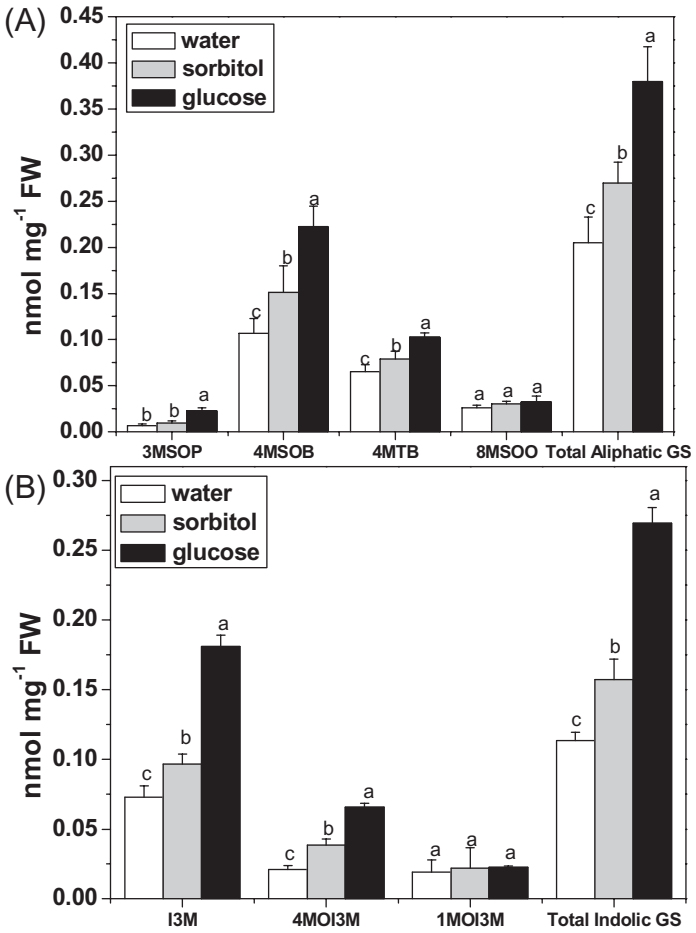

Fig. 3. Effect of glucose on accumulation of individual and total aliphatic and indolic glucosinolates. (A) Total aliphatic glucosinolates and four aliphatic glucosinolates 3MSOP, 4MSOB, 4MTB, and 8MSOO (8-methylsulphinyloctyl glucosinolate). (B) Total indolic glucosinolates and three indolic glucosinolates I3M, 4MOI3M, and 1MOI3M (1-methoxyindol-3-ylmethyl) were measured in 10-day-old *Arabidopsis* seedlings treated with 3% glucose and sorbitol. Samples were collected 3 d after treatment. Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$.

(Supplementary Fig. S3 at *JXB* online). Furthermore, glucose treatment did not rescue the chemotype of low glucosinolates in the mutant plants. The total aliphatic glucosinolate content was significantly lower in *gin2-1* and *rgs1-2* plants than that in the control under glucose treatment. These results indicate that HXK1 and RGS1 are involved in the glucose-regulated aliphatic glucosinolate biosynthesis.

Considering that HXK1 controls gene transcription via interaction with several protein families including MYB in the nucleus (Rolland *et al*., 2006), the expression level of three transcription factors involved in aliphatic glucosinolate biosynthesis (*MYB28*, *MYB29*, and *MYB76*) was investigated in *gin2-1* mutant plants. Although glucose treatment increased the expression of *MYB28* and *MYB29* in the *gin2-1* mutant when compared with sorbitol treatment (Fig. 6C, D), severely lower expression levels of *MYB28*, *MYB29*, and *MYB76* (Fig. 6C, D, E) were found in *gin2-1* as compared with L*er* after glucose treatment.

Effect of glucose on aliphatic glucosinolate biosynthesis in the abi5 mutant

A significant decrease in total aliphatic glucosinolates was observed in the *abi5-7* mutant in comparison with the wild type with or without glucose treatment (Fig. 7A). Furthermore, expression levels of *MYB28*, *MYB29*, and *MYB76* in *abi5-7* were substantially lower than that in the wild type after glucose treatment (Fig. 7B–D).

Discussion

Glucose-induced aliphatic glucosinolate accumulation

Sugars were first recognized to be the prime carbon supply and energy source in plants. However, reports on their regulatory functions have been increasing in recent years (Smeekens *et al*., 2010). The regulatory mechanism of sucrose in anthocyanin biosynthesis has been well elucidated (Teng *et al*., 2005). In addition, previous studies indicated that sugars could boost the accumulation of health-promoting compounds in *Brassica* vegetables (Guo *et al*., 2011; Wei *et al*., 2011). As a signalling molecule, glucose controls plant growth, development, metabolism, and stress resistance (Ramon *et al*., 2008). In the current study, the results indicated that glucose positively regulated aliphatic glucosinolate biosynthesis. Aliphatic glucosinolates can be greatly induced through MYB transcription factors by glucose in a time- and dose-dependent manner (Figs 1, 4), while aliphatic glucosinolate accumulation was severely disrupted in the glucose signalling mutants *gin2-1* and *rgs1-2* (Fig. 6A, B). In addition, because glucosinolate contents could be affected by several abiotic stress factors, such as salt, drought, and water (López-Berenguer *et al*., 2008; Khan *et al*., 2010; Yuan *et al*., 2010), sorbitol was taken as an osmotic stress control and its effect on glucosinolates was not as strong as that of glucose. Moreover, fructose, the isomer of glucose, which can interconvert with glucose and enter the metabolic pathway of glucosinolates with the form of glucose-6-phosphate as intermediate (Henry *et al*., 1991; Bhagavan and Ha, 2011), could not mimic the induction by glucose of glucosinolate accumulation (Supplementary Fig. S4 at *JXB* online). In addition, the glucose analogue mannose, the substrate of HXK1 phosphorylation, which can also trigger the signalling function of HXK1 (Ramon *et al*., 2008), did not have a similar induction to glucose (Supplementary Fig. S5). To sum up, glucose induces aliphatic glucosinolate accumulation as a signalling molecule in *Arabidopsis thaliana* through a mechanism different from mannose.

Induction of aliphatic glucosinolates by glucose through MYB transcription factors

MYB28 and MYB29 are the two vital transcription factors in aliphatic glucosinolate biosynthesis in *A. thaliana*

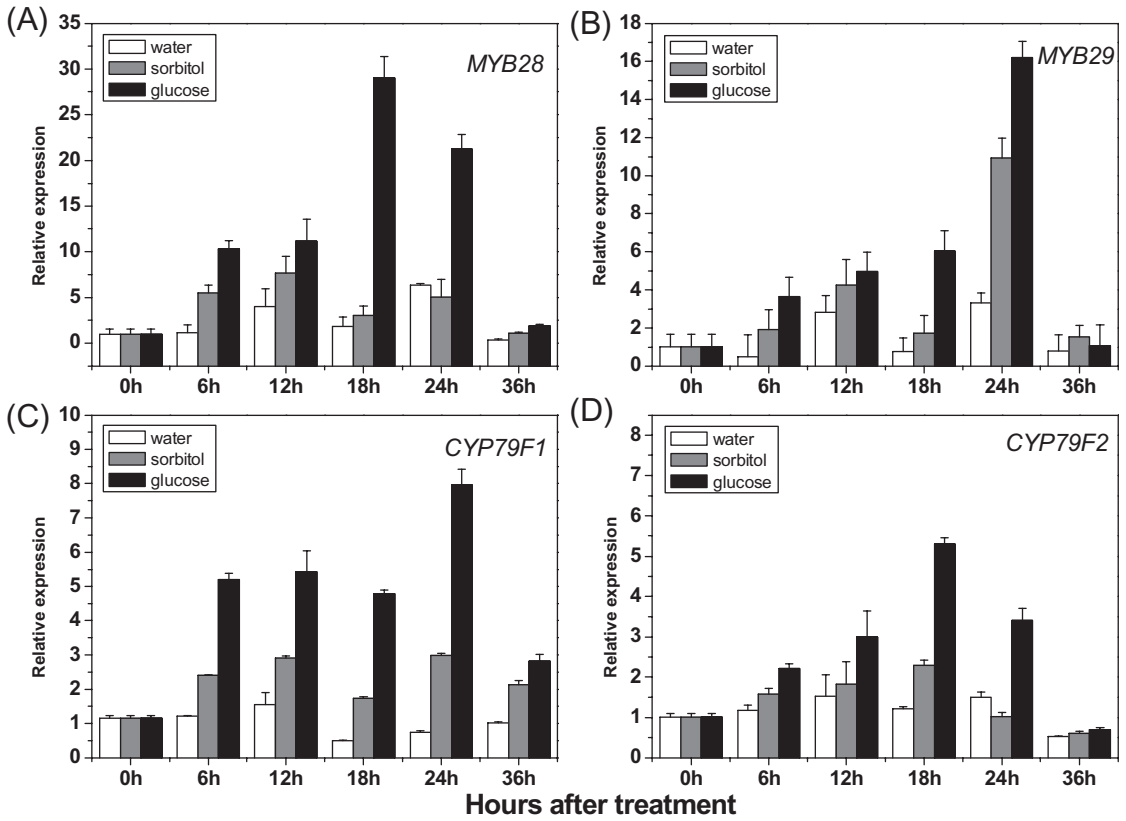

Fig. 4. Effect of glucose on the expression level of *MYB28* (A), *MYB29* (B), *CYP79F1* (C), and *CYP79F2* (D). The expression level was measured in 10-day-old *Arabidopsis* seedlings treated with 3% glucose or sorbitol. Samples used for qPCR experiment were collected 0, 6, 12, 18, 24, and 36 h after treatment, respectively. Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Relative expression values are given compared with 0 h non-treated seedlings (0 h=1).

(Gigolashvili *et al*., 2009). Almost all the biosynthetic genes in the aliphatic glucosinolate pathway can be positively regulated by these two transcription factors (Yan and Chen, 2007). To test whether aliphatic glucosinolate biosynthesis induced by glucose was due to the up-regulation of MYB transcription factors, qPCR was applied to determine the expression pattern of *MYB28* and *MYB29* after glucose treatment. As expected, induction of these two genes was similar at different time points except that the expression of *MYB28* had an earlier peak than that of *MYB29*. This exactly reflected that MYB28 could regulate the expression of *MYB29* (Yatusevich, 2008) (Fig. 4A, B). In addition, the expression levels of *CYP79F1* and *CYP79F2*, the key structural genes in aliphatic glucosinolate biosynthesis, were also enhanced after glucose treatment (Fig. 4C, D). The induction occurred within the first 6 h, which was a little earlier than glucosinolate enhancement. This indicates that the expression of transcription factors can sense changes in the environment in a shorter time than the production of secondary metabolites. The results are consistent with previous reports on *Brassica* crops with sucrose treatment (Guo *et al*., 2011; Wei *et al*., 2011).

To obtain a deeper understanding of the role that the main transcriptional regulators and structural genes played in the regulation of aliphatic glucosinolate biosynthesis in response to glucose, three mutants, *myb28*, *myb29*, and *myb28myb29*, were used for further analysis. MYB28 is the predominant transcription factor belonging to the MYB family controlling the profiles of aliphatic glucosinolates, and it is mainly responsible for long-chain and short-chain aliphatic glucosinolates, while MYB29 participates more in short-chain aliphatic glucosinolates. In the present study, *myb28* showed a very severe lack of aliphatic glucosinolates compared with *myb29* (Hirai *et al*., 2007; Gigolashvili *et al*., 2008; Yatusevich, 2008) (Fig. 5A). Surprisingly, the reduction could be recovered by glucose supplementation in *myb29* but not in the case of *myb28* (Fig. 5A). This is due to the fact that these two transcription factors have functional redundancy in regulation of aliphatic glucosinolate, in which MYB28 takes a master role while MYB29 has an accessory role (Hirai *et al*., 2007). According to previous reports (Yatusevich, 2008), *MYB29* can be induced by MYB28. Thus, when *myb29* was treated with glucose, the strong expression of *MYB28*, induced by glucose, could compensate the decreased level of *MYB29* expression (Fig. 5B, C). When *MYB28* is knocked out, the expression of *MYB29* is still lower than that in Col-0 even under glucose treatment (Fig. 5C). Interestingly, the induction by glucose of total aliphatic glucosinolates and the expression of *MYB29* in mutant *myb28* were much weaker than that in Col-0. This observation indicated that the strong induction by glucose of the expression of *MYB29* in Col-0 is

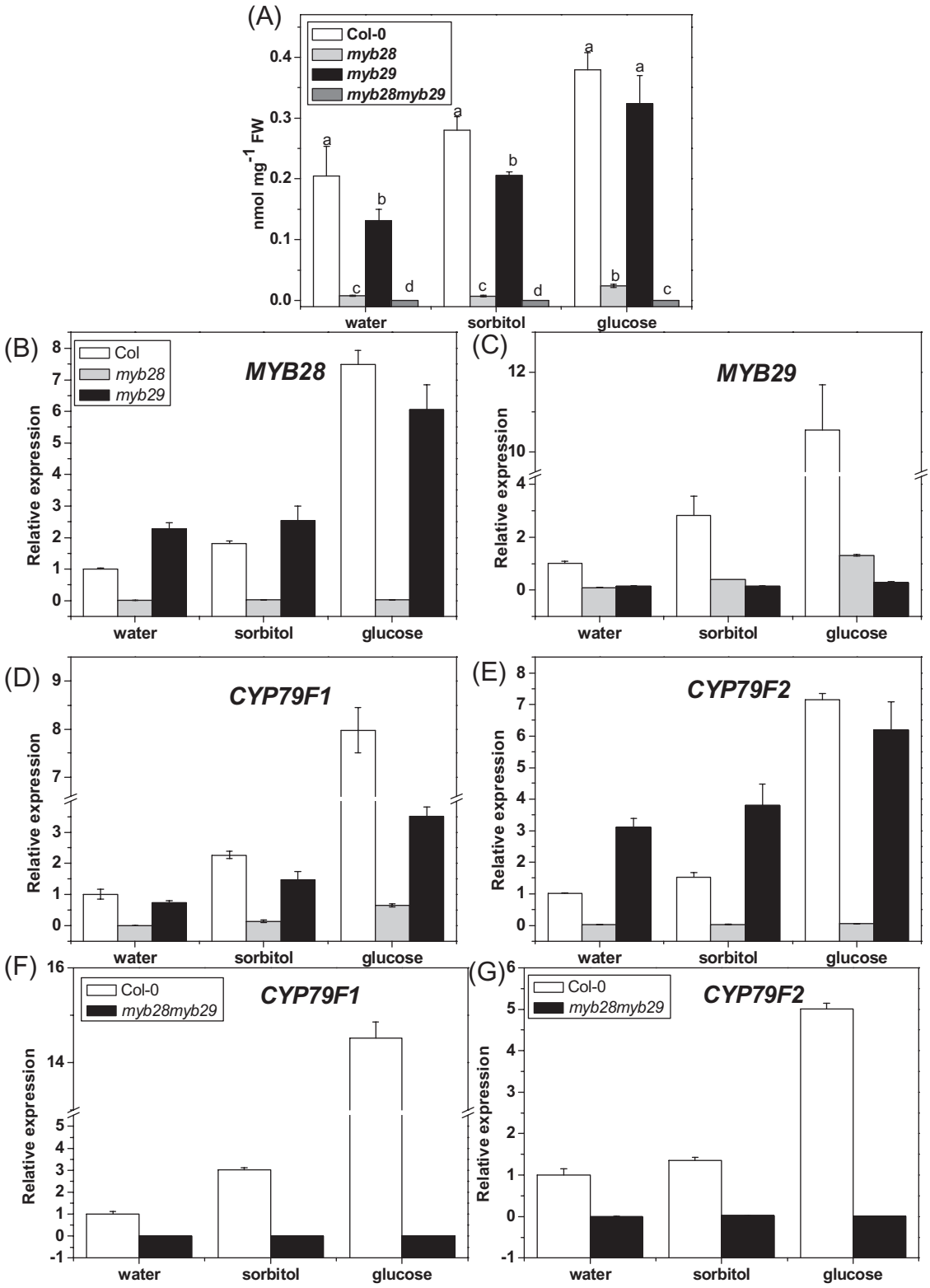

Fig. 5. Total aliphatic glucosinolates content of *myb28* and *myb29* (A). Effect of glucose on *MYB28* (B), *MYB29* (C), *CYP79F1* (D), and *CYP79F2* (E) expression level in *myb28* and *myb29*, and of *CYP79F1* (F), *CYP79F2* (G) in the *myb28myb29* double mutant. Samples used for glucosinolate assay were collected 3 d after treatment. Samples used for qPCR experiment were collected 18 h after treatment. Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$. Relative expression values are given compared with seedlings treated by water.

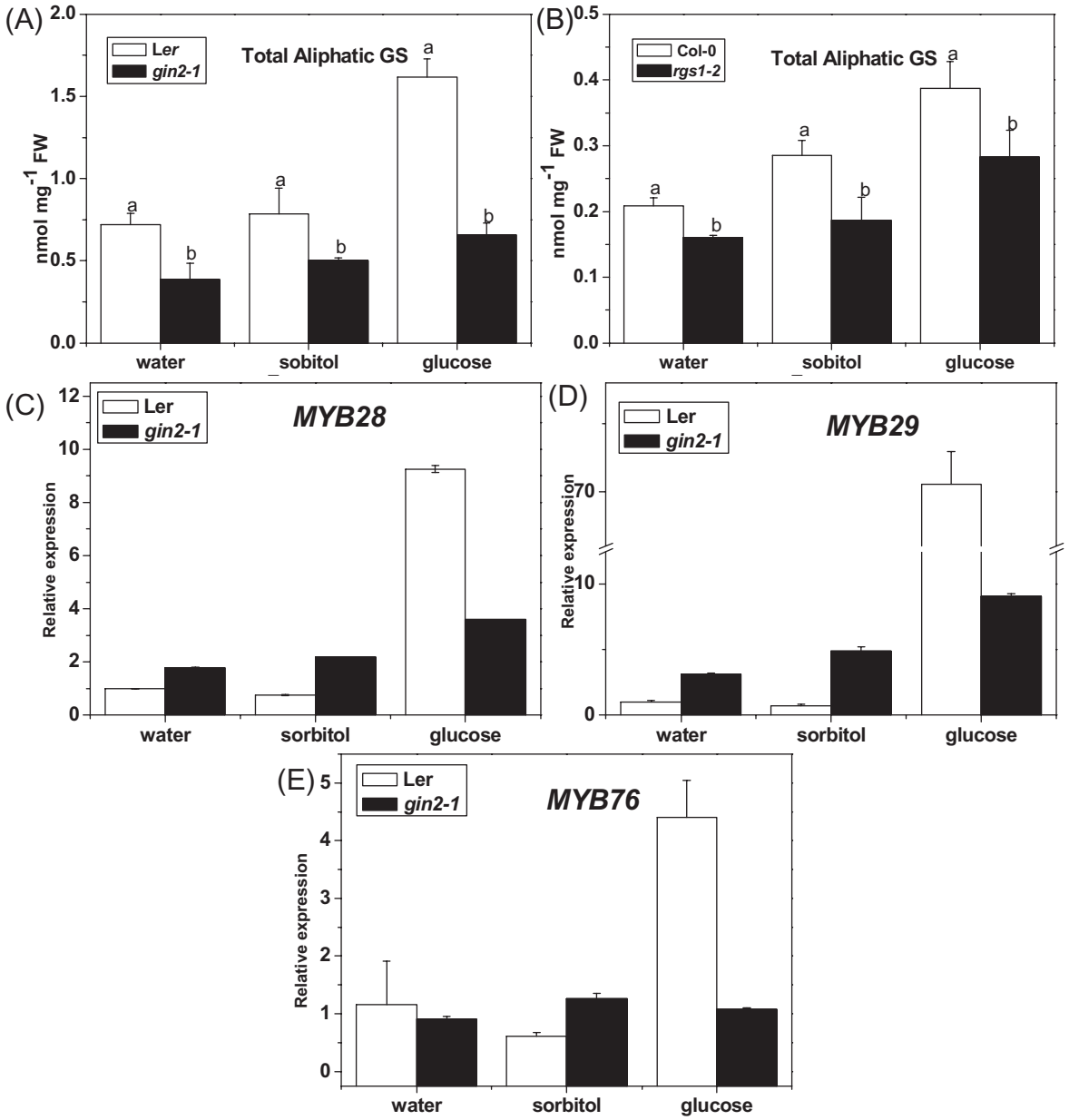

Fig. 6. Total aliphatic glucosinolate content of *gin2-1* and *rgs1-2* (A, B). Effect of glucose on the *MYB28*, *MYB29*, and *MYB76* expression level in the *gin2-1* mutant (C, D, E). *Arabidopsis* seedlings (10-day-old) were treated with 3% glucose or sorbitol. Samples used for glucosinolate assay were collected 3 d after treatment. Samples used for qPCR experiment were collected 18 h after treatment. Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$. Relative expression values are given compared with seedlings treated by water.

partly due to the regulation of MYB28 (Fig. 4). These results may explain why *myb28* contained a significantly reduced level of aliphatic glucosinolates with or without glucose treatment.

Aliphatic glucosinolates and the expression of *CYP79F1* and *CYP79F2* could not be detected in the loss-of-function mutant *myb28myb29* (Fig. 5F, G), which is consistent with previous surveys (Sønderby *et al.*, 2007). Interestingly, it contained no detectable aliphatic glucosinolates even when treated with glucose. Previous research has illustrated that *CYP79F1* and *CYP79F2* could be regulated by MYB factors (MYB28, MYB29, and MYB76) (Gigolashvili *et al.*, 2008). Here the up-regulated expression of *CYP79F1* and *CYP79F2* in Col-0 was not observed in *myb28myb29* before and after glucose treatment. These findings suggest that the MYB transcript factors are induced by glucose, and then regulate the expression of structural genes which finally lead to the accumulation of aliphatic glucosinolate.

HXK1- and RGS1-dependent regulation of aliphatic glucosinolate biosynthesis by glucose

HXKs are one of the most conserved sugar sensors together with other sugar kinases in the plant kingdom. In *A. thaliana*, six HXK and HXK-like genes have been identified so far

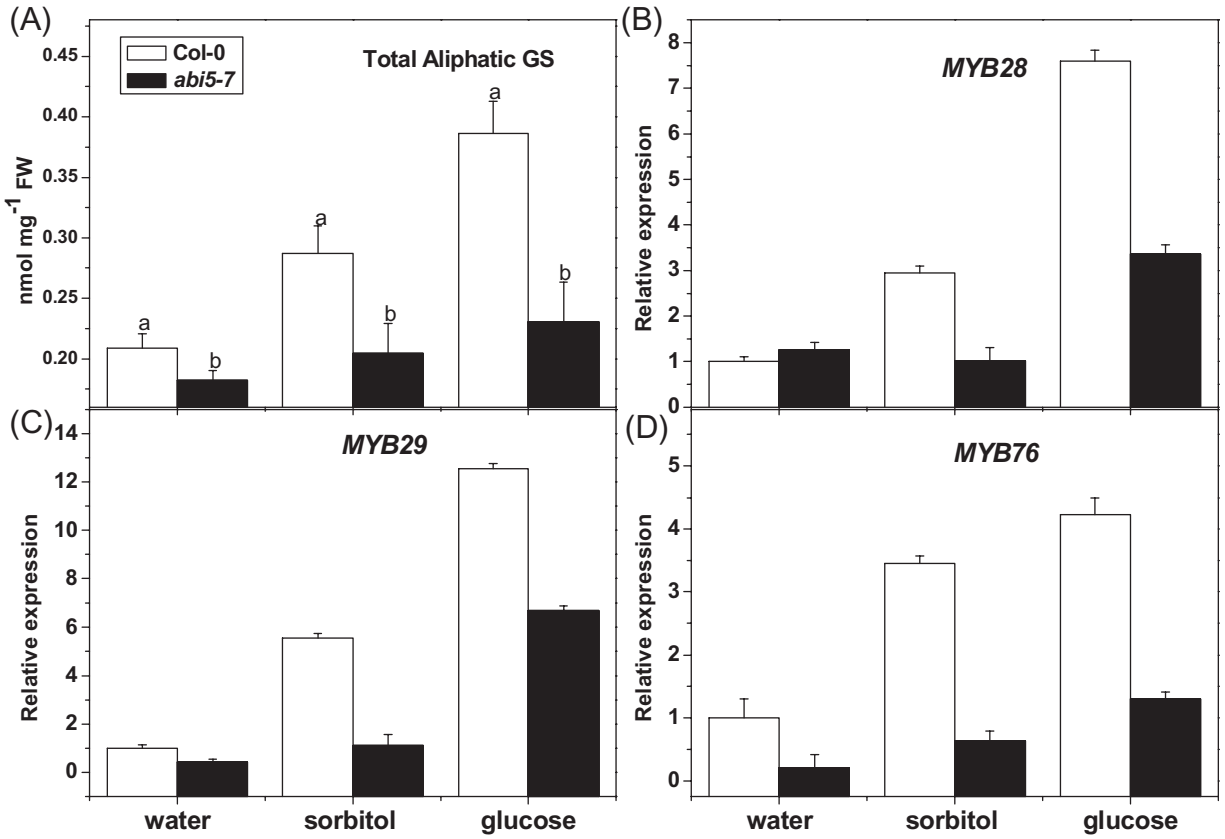

Fig. 7. Total aliphatic glucosinolate content and effect of glucose on the *MYB28*, *MYB29*, and *MYB76* expression level in *abi5-7*. *Arabidopsis* seedlings (10-day-old) were treated with 3% glucose or sorbitol. (A) Samples were collected 3 d after treatment. (B–D) Samples were collected 18 h after treatment. Each data point represents the mean of three independent biological replicates per treatment (mean ±SE). Values not sharing a common letter are significantly different at $P < 0.05$. Relative expression values are given compared with seedlings treated by water.

(Ramon *et al*., 2008) and they carry out diverse and distinct functions in glucose metabolism and signalling. AtHXK1 has been characterized as an intracellular glucose sensor (Bolouri-Moghaddam *et al*., 2010). HXK1-dependent glucose signalling can affect plant growth, which relies on the endogenous glucose level and the sensitivity to glucose in plants (Ramon *et al*., 2008). In addition, there are other glucose-sensing and signalling pathways independent of HXK1. It has been reported that the signalling pathway of sugar-induced anthocyanin accumulation is independent of HXK1 (Xiao *et al*., 2000; Teng *et al*., 2005). Furthermore, the fructose signalling can function independently of the HXK1 glucose sensor (Li *et al*., 2011). In *A. thaliana*, RGS1 is a regulator of G-protein signalling protein, which modulates plant cell proliferation and may serve as a glucose sensor located on the cell surface, independent of HXK1 (Chen and Jones, 2004; Ramon *et al*., 2008).

In the present study, the relationship between HXK1/RGS1 and aliphatic glucosinolates was analysed using their corresponding mutants, *gin2-1* and *rgs1-2*. Both mutants are glucose insensitive. According to the present results, *gin2-1* and *rgs1-2* mutants showed a notable decrease in the level of total aliphatic glucosinolates whether treated with glucose or not, when compared with their corresponding controls (Fig. 6A, B). This suggested that HXK1 and RGS1 might both participate in the regulation of aliphatic glucosinolate biosynthesis. It has been reported that HXK1 regulates transcription and proteasome-mediated degradation of the EIN3 (ETHYLENE INSENSITIVE3) transcription factor in the nucleus, and several types of transcription factors are involved in sugar-regulated transcription (Rolland *et al*., 2006; Ramon *et al*., 2008). Therefore, the gene expression pattern of three MYB transcription factors involved in aliphatic glucosinolate biosynthesis (*MYB28*, *MYB29*, and *MYB76*) was analysed in *gin2-1* mutants, and a substantial decrease in the expression level in response to glucose was observed when compared with the wild type (Fig. 6C–E). This is undoubtedly another piece of evidence for HXK1-dependent induction of aliphatic glucosinolate biosynthesis by glucose signalling through MYB factors.

ABI5 is involved in the regulation of aliphatic glucosinolate biosynthesis by glucose

Previous studies have indicated that glucose signalling is a complex network, and cross-talk between glucose signalling and phytohormone signalling was elucidated (Gazzarrini and McCourt, 2001; Dekkers *et al*., 2008). ABA signalling and sugar sensing share several common components, and

many glucose-insensitive mutants are also allelic to ABA biosynthetic or signalling mutants (Finkelstein and Gibson, 2002; Rook *et al*., 2006). *ABI5* (ABA-insensitive 5) encodes a transcription factor belonging to a large basic leucine zipper (bZIP) gene family, conferring on *abi5-7* a glucose-insensitive phenotype. ABI5 is therefore a putative glucose signalling component downstream of HXK1 (Ramon *et al*., 2008; Reeves *et al*., 2011). In this study, *abi5-7* was deficient in total aliphatic glucosinolate content before and after glucose treatment when compared with the wild type, as well as the expression level of MYB transcription factors after glucose treatment (Fig. 7). These results suggested that ABI5 might be involved in the regulation of aliphatic glucosinolate biosynthesis as a glucose signalling component downstream of HXK1.

In conclusion, glucose induces the accumulation of aliphatic glucosinolate as a signalling molecule by modulating MYB transcription factors (MYB28 and MYB29), which participated in the regulation of aliphatic glucosinolate biosynthesis, with MYB28 as a master component. Furthermore, two distinct glucose sensors, HXK1 and RGS1, as well as ABI5, a putative glucose signalling component located downstream of HXK1, are proved to be involved in the network of glucose signalling to regulate aliphatic glucosinolate biosynthesis. The abolishment of increased expression of *MYB28*, *MYB29*, and *MYB76* induced by glucose in *gin2-1* and *abi5-7* mutants in the present study indicates an interaction between HXK1 or ABI5 and the MYB factors involved in regulation of aliphatic glucosinolate biosynthesis.

Further analysis of the interplay between HXK1/ABI5 and *MYB28*/*MYB29*/*MYB76* will help to elucidate the regulatory mechanism of aliphatic glucosinolate biosynthesis induced by glucose signalling. Another possibility of glucose enhancing sulphate assimilation should also be considered. Glucosinolates are sulphur-rich plant metabolites, and sulphur fertilization usually causes an increase in glucosinolate content (Falk *et al*., 2007). Previous reports showed that sugars (sucrose and glucose) at low concentration induced APR (adenosine 5-phosphosulphate reductase) activity and enhanced sulphate uptake (Kopriva *et al*., 2002; Hesse *et al*., 2003; Kopriva, 2006). Therefore, further investigation is needed to elucidate whether glucose induces the accumulation of glucosinolates by regulating sulphate assimilation and uptake.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Effect of glucose on accumulation of total aliphatic glucosinolates with the solid culture method.

Figure S2. Effect of glucose on accumulation of total aliphatic glucosinolates during 36 h after treatment.

Figure S3. Total content of aliphatic glucosinolates of mutants *gin2-1* and *rgs1-2* grown on solid culture medium.

Figure S4. Effect of fructose on the accumulation of total aliphatic glucosinolates.

Figure S5. Effect of mannose on the accumulation of total aliphatic glucosinolates.

Figure S6. Effect of glucose on the accumulation of total aliphatic glucosinolates in *Arabidopsis* cultured in half-strength MS salt solution.

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