Sugar regulation of SUGAR TRANSPORTER PROTEIN 1 (STP1) expression in Arabidopsis thaliana

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Abstract

Sugars regulate the expression of many genes at the transcriptional level. In Arabidopsis thaliana, sugars induce or repress the expression of >1800 genes, including the STP1 (SUGAR TRANSPORTER PROTEIN 1) gene, which encodes an H+/monosaccharide cotransporter. STP1 transcript levels decrease more rapidly after the addition of low concentrations of sugars than the levels of other repressed genes, such as DIN6 (DARK-INDUCED 6). We found that this regulation is exerted at the transcriptional level and is initiated by phosphorylatable sugars. Interestingly, the sugar signal that modulates STP1 expression is transmitted through a HEXOKINASE 1-independent signalling pathway. Finally, analysis of the STP1 5′ regulatory region allowed us to delimit a region of 309 bp that contains the cis elements implicated in the glucose regulation of STP1 expression. Putative cis-acting elements involved in this response were identified.

Key words: Arabidopsis, STP1, sugar repression, sugar signalling, sugar transporter, transcriptional regulation.

Introduction

As autotrophic organisms, plants produce their carbon skeletons through the photosynthetic process in the form of sugars. These carbon skeletons are essential as structural components and energy sources for plant growth and development. Similarly to many other organisms, plants respond to the carbon fluctuations caused by changes in photosynthetic efficiency or metabolic status and adjust their growth and development accordingly (Baena-Gonzalez and Sheen, 2008; Nunes-Nesi et al., 2010; Eveland and Jackson, 2012). Sugars act as signalling molecules, and plants have evolved mechanisms to efficiently perceive sugar availability and respond by modulating gene expression and protein activity in response to their nutrient status (Gibson, 2005; Rolland et al., 2006; Smeekens et al., 2010). The presence of sugars induces different developmental programmes, including growth, starch biosynthesis, and cell division. In contrast, sugar starvation upregulates photosynthetic activities and carbon remobilization, thus affecting central aspects of development (Koch, 1996; Borisjuk et al., 2003). Thus, understanding the mechanisms involved in sugar perception form an important area of research.

Plants have the capacity to sense different sugars, including sucrose, hexoses, and trehalose, and elicit responses, some that are specific to the type of sugar (Chiou and Bush, 1998; Sheen et al., 1999; Eastmond and Graham, 2003; Price et al., 2004; Wind et al., 2010). However, hexoses appear to be the most common signal detected by plants (Rolland et al., 2006; Smeekens et al., 2010). Diverse lines of evidence have demonstrated that sugar levels are detected by specific receptors and through independent signalling pathways (Rolland et al., 2006; Hanson and Smeekens, 2009). One of those receptors is the HEXOKINASE 1 (HXK1) that, in addition to its enzymatic activity, acts a sugar sensor (Jang et al., 1997; Moore et al., 2003; Cho et al., 2006). Experimental evidence
has shown that upon sugar phosphorylation, HKX1 interacts with the VHA-B1 and RPT5B proteins to control the transcription of an important number of target genes (Cho et al., 2006). Evidence exists for additional sugar receptors, including sugar transporters and the negative regulator of trimeric G-protein (RGS1), but their mechanisms of action and downstream components are still poorly understood (Chen and Jones, 2004; Rolland et al., 2006).

Some of these sensors and downstream components of the sugar signalling pathways have initially been identified through genetic approaches with the isolation of sugar-resistant mutants. The characterization of these mutants has demonstrated the complexity of sugar signalling and the extensive cross-talk with other signalling pathways (León and Sheen, 2003; Gibson, 2005; Rolland et al., 2006; Eveland and Jackson, 2012). Additional components that are required for proper sugar perception were identified from sugar-insensitive mutants, including the enzyme ABAR2, the transcription factors AB14 and AB15, and the ethylene-insensitive EIN2 protein (Zhou et al., 1998; Arenas-Huertero et al., 2000; Cheng et al., 2002). These factors were originally identified as components of the abscisic acid (ABA) or ethylene biosynthesis and signalling pathways, demonstrating the cross-talk between sugars and these hormones (Finkelstein and Gibson, 2002; León and Sheen, 2003; Yamagishi et al., 2009). Cross-talk has also been reported between sugar signalling and other hormones, such as auxin and gibberellins (Moore et al., 2003; Eveland and Jackson, 2012). Sugar signalling not only interacts with hormones but also with other nutrients, such as nitrogen (Coruzzi and Bush, 2001) and with the energy and stress signalling responses through the participation of the SnRK1 and TOR complexes (Baena-Gonzalez et al., 2007; Baena-Gonzalez, 2010; Xiong et al., 2013).

Based on the genes regulated by different sugars and sugar analogues, several pathways for sugar signalling are recognized and can be grouped into those that depend on HKX1 for signal initiation and those that are independent of this sensor (Rolland et al., 2006). The last group includes several pathways, such as the SnRK1-mediated pathway (Baena-Gonzalez et al., 2007; Jossier et al., 2009), the RGS1 pathway and most likely other undiscovered pathways (Chen and Jones, 2004; Chen, 2008; Sheen, 2010). Due to the complexity of sugar signalling, alternative strategies are required to further understand the molecular basis and additional components of the different sugar signalling pathways.

The regulation of gene expression is one of the most prominent mechanisms by which sugars modulate a variety of responses in plants. Independently of the signalling pathway, sugars positively or negatively affect the transcription of nearly 2000 different genes (Wang et al., 2003; Price et al., 2004; Li et al., 2006; Müller et al., 2007). In spite of the number of genes regulated by sugars, only a few transcriptional factors are known to be involved in this regulation. In fact, the participation of several bZIP and MYB transcription factors was recognized through the use of novel screenings (Rolland et al., 2006; Hanson and Smeekens, 2009; Sheen, 2010). The analysis of target genes has also proven to be a useful approach for identifying the cis-acting elements and trans-acting factors that are involved in sugar regulation. For example, using the promoter region of the α-amylase (α-Amy3) gene from rice allowed the identification of different MYB transcription factors that participate in the sugar regulation mediated by SnRK1A (Lu et al., 2007).

To further elucidate the mechanisms underlying sugar signalling in plants, we characterized the regulation by sugars of the Arabidopsis thaliana STP1 gene (AT1G11260). STP1 encodes a high-affinity sugar transporter that acts as an H+/monosaccharide cotransporter, capable of transporting a wide range of hexoses (Boorer et al., 1994; Büttner and Sauer, 2000). STP1 belongs to a family of 14 members that are highly conserved among plants and mediate hexose transport in cells of different tissues (Stadler et al., 2003; Slewinski, 2011). Several of these transporters are expressed in a tissue-specific manner, or at specific developmental stages (Büttner, 2010). STP1 is the member of the STP family with the highest expression level (Johnson et al., 2006; Johnson and Thomas, 2007). This high expression is detected in photosynthetic tissues, such as leaves and stems, while roots, siliques, and flowers show lower expression levels (Sherson et al., 2003). The expression of this transporter was also detected in guard cells and its accumulation responds to diurnal fluctuation that correlates with the accumulation of sucrose in this cell type. This has led to speculation that this H+/sucrose cotransporter might be important for osmoregulation during the day and night periods (Stadler et al., 2003). The expression of several members of the STP family, including STP1, STP4, STP13, and STP14, is strongly repressed by sugars, and STP1 is one of the most repressed genes, as indicated by wide-genome analyses (Price et al., 2004; Büttner, 2010). However, neither the pathway implicated in this regulation nor the factors involved are known. In this work, we analysed the mechanism that regulates the expression of the STP1 gene in response to sugar levels. This analysis demonstrated that the STP1 transcript is strongly downregulated within minutes after sugars increase. Interestingly, the regulation of this gene by sugars depends on phosphorylated hexoses but is independent of HKX1. We demonstrated that the regulation of this gene occurs at the transcriptional level and that the cis-acting elements responsible for this regulation are within a 309bp fragment of the promoter.

Material and methods

Plant material and growth conditions

For sterile growing conditions, A. thaliana seeds were sterilized following standard protocols (http://www.arabidopsis.org). To break dormancy, the seeds were incubated for 3 days at 4°C in darkness. Adult plants were grown in Metro-Mix 200 (Grace Sierra, Milpitas, CA, USA). Plants and seedlings were grown under a 16h light/8h dark cycle in 120 μM m⁻² s⁻¹ light conditions at 22°C. Wild-type Col-0 and the gin2, abi4-1, abi5, kin11, rgs1, and rgs1-2 mutants were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). The KIN10 knockout mutant and overexpressing lines were kindly donated by Dr Phillip Rolland (Institute of Botany and Microbiology, Heverlee-Leuven, Belgium). For sugar gene expression analysis, 50 seeds per treatment were grown in liquid 0.1X GM medium containing Murashige and Skoog basal salts (Caisson Laboratories Inc., UT, USA), supplemented with B5.
vitamins (Sigma In., MO, USA), 0.05% MES, and 0.5% sucrose, and maintained with agitation at 350 rpm. After 10 d, this medium was replaced with carbon (-C) starvation medium (0.1X GM without sucrose) for 2 d. Finally, the treatments were applied in 0.1X GM with or without sugar as indicated in each case using D-glucose monohydrate (Research Organics Cleveland, USA) or mannitol (Sigma-Aldrich, MO, USA) as carbon sources, as indicated.

Transgenic lines were generated through Agrobacterium tumefaciens-mediated transformation by floral dipping (Clough and Bent, 1998) into the Col-0 ecotype. Transgenic lines were selected in 1X GM media with 0.8% Phytagar and supplemented with 50 µg ml⁻¹ kanamycin. At least three independent lines were selected for each construct.

**Plasmid constructions**

To generate transcriptional fusions of the *STP1* upstream region with the GUS reporter gene (Jefferson et al., 1987), the 2.4 kb fragment of the intergenic region of *STP1* (between the loci AT1G11250 and AT1G11260) was amplified by PCR from DNA using the oligonucleotides STP1-3, 5'-AAG GCT TCT CAT TCT AGG AAT TCA AAT CAA ACA GGA CCC GTG AA-3' and STP1-5R, 5'-GGA TCA TAC ACA AGA CCC GTG AAA C3-'. The 1.3 kb, 1 kb, and 0.5 kb deletions were generated from the original 2.4 kb fragment through PCR using the following specific forward oligonucleotides: STP1-1327F, 5'-CCA ATG CGG CCC ATG AAA C3-'; STP1-1HF, 5'-GGT GAA GGT TAA GAC TAT G-3'; and the common reverse STP1-5R oligonucleotide. All the fragments were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. Each fragment was subcloned into the pBI101 vector binary vector (Clontech Laboratories, Inc. CA, USA) in the HindIII and BamHI restriction site.

**Expression analysis**

Total RNA was isolated from frozen tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. For northern blot analyses, 1–20 µg of total RNA was fractionated in 1.5% agarose denaturing gels with 2% formaldehyde (Mallinckrodt Baker, MEX) and transferred onto a Hybond-N⁺ nylon membrane (GE, Bucks, UK). Hybridizations and washes were performed in stringent conditions. Probes were ³²P-radio labelled using the Megaprime DNA labelling system, according to the manufacturer’s protocol (GE, Bucks, UK). All probes were obtained by PCR amplification from DNA or cDNA as indicated. The *STP1* (At1g11260) probe corresponds to a cDNA fragment of 499 bp that was obtained using the oligonucleotides STP1-1, 5'-TGG TAT AGT GGT TGT AAC GCT CAT T-3', and STP1-2, 5'-GTC TAA TAC ACT ATC TCC TTT ACG A3-'. The GUS probe (860 bp) was obtained using the oligonucleotides GUS3F, 5'-GCC TGC TCG AAC TCT GAA AGA-3' and GUS4R, 5'-GAC ATT GCC AGG TTC AAT GAA-3'. For *DIN6* (At3g47340), a 382 bp fragment was obtained using the oligonucleotides DIN6F, 5'-GCC TGA AAG ATC AGC TCT AGG GAT G-3' and DIN6R, 5'-GCC TGT GAA GGT GAA CAA GGC-3'. For β-*AMY*1 (At4g15210), a 608 bp cDNA fragment was obtained using the oligonucleotides β-Amy1-1, 5'-CTG AGA AGG GGA AGT TTT TCC T-3', and β-Amy1-2, 5'-ATT CCT ATG CCC GTG TAT CG-3'. For *SBE2* (At3g03650), a 335 bp cDNA fragment was obtained using the oligonucleotides SBE2.2A, 5'-GAG TGT CTC TTA TTA TTC CAC GC-3' and SBE2.2B, 5'-GGG AAC TAT TGT TGG TTT CAC-3'. For *APL3* (At4g39210), a 345 bp cDNA fragment was obtained using the oligonucleotides Apl3-1, 5'-GTT GAA GGA AAA TGC ACC ATC-3', and Apl3-2, 5'-GTA TCA TAT CAC AGC ACC GTC-3'. Densitometric analysis was performed using the Image J 1.43u program from the National Institutes of Health, USA, http://rsb.info.nih.gov/ij/. To evaluate confidence of the data we used ANOVA statistical analysis (http://www.r-project.org/).

**GUS histochemical analysis**

Twelve-day-old seedlings exposed to different sugar sources for 12 h were stained for 2h using the GUS histochemical assay as reported (Jefferson et al., 1987). Plant were clarified using a modified protocol from Malamy and Benfey (1997). Pigments were removed with 70% ethanol and plants were rehydrated by incubations in 50% and 30% ethanol for 15 min each, and then transferred to a solution of 0.24 N HCl in 20% methanol and incubated at 62°C for 1h. This solution was replaced by 7% NaCl in 60% methanol and incubated for 25 min at room temperature. Then plants were dehydrated with 40%, 20%, and 10% ethanol for 15 min each to finally be mounted in a solution with 50% glycerol and 2% DMSO. Samples were visualized using a stereoscopic (Nikon SMZ1500) and a light microscope (Nikon Eclipse E600).

**In silico analysis**

The 309 bp sequence from the *STP1* promoter was analysed using the PLACE (Plant cis-acting Regulatory DNA Elements) database (Prestridge, 1991; Higo et al., 1999), and released data were analysed to identify the reported motifs involved in sugar regulation. Additional cis elements, which were not included as sugar-responsive elements in this database, were identified by manual comparison with the promoter sequence for *DIN6* (At3g47340), a gene that is downregulated in the presence of sugar (Li et al., 2006; Baena-Gonzalez et al., 2007).

**Results**

**STP1 expression is rapidly regulated by glucose supply**

Analysis of the available microarray data indicated that *STP1* (AT1G11260) was one of the most prominent downregulated genes in response to sugars in *A. thaliana* (Price et al., 2004). Thus, we wanted to characterize the regulatory mechanism involved in the *STP1* gene response to sugars. To corroborate the effect of sugars on *STP1* expression, we analysed the accumulation of its transcript in the presence of glucose (Glc) by northern blotting. Sugar treatments were performed after carbon starvation in liquid media (see Material and Methods). As shown in Fig. 1A and in agreement with published microarray data, the *STP1* transcript level was dramatically reduced in the presence of 150 mM Glc, relative to the levels in the untreated samples (–C) or in the isosmotic control with mannitol (Mtl). Under these conditions, the expression was induced (Fig. 1A) for the known Glc upregulated genes, such as β-*AMY* (Beta-amylase) and *SBE2.2* (Starch Branching Enzyme 2.2), that were used as controls (Rook et al., 2001). These results confirm that the *STP1* transcript is downregulated by the presence of Glc (Price et al., 2004).

For more detailed analysis of the regulation of the *STP1* gene in response to sugar, the level of its transcript was followed at different times after the addition of Glc. As shown in Fig. 1B, the *STP1* transcript level decreased 15 min after the addition of Glc. This repression was not observed in the addition of Mtl. Under these conditions, the transcript was basically undetectable 1 h after the treatment. The rapid response of *STP1* contrasts with the slower response for other characterized sugar-repressed genes, such as *DIN6/ASN1*, which encodes the asparaginase synthetase 1 enzyme (Lam et al., 1998; Price et al., 2004).

The reduction of the *DIN6* transcript was evident only 3h
after the addition of Glc (Fig. 1B). A similar situation was observed for the Glc-upregulated APL3 gene (encodes the large subunit of ADP-glucose pyrophosphorylase), whose transcript accumulation in response to Glc was detectable only 3 h after the addition of sugar (Fig. 1B). These findings demonstrate that the expression of STP1 is rapidly modulated by the changes in sugar levels.

To establish the sensitivity of STP1 to sugars, the expression of this gene was analysed in the presence of different Glc concentrations (data not shown). As shown in Fig. 1C, the presence of 5 mM Glc was sufficient to dramatically decrease STP1 transcript levels, albeit after a longer time (1 h) than the 15 min that was found with 150 mM Glc (Fig. 1B). This reduction was not observed with 3-O-methylglucose (3-OMG), a poorly metabolized Glc analogue (Fig. 1C). These data allowed us to conclusively demonstrate that the accumulation of the STP1 transcript is rapidly downregulated by the presence of low Glc levels and that this regulation is not related to an osmotic response.

**STP1 gene expression is dynamically regulated by sugar fluctuation**

Experimental evidence has demonstrated that regulation by sugars is complex. For example, sugar regulation of the \( \alpha \)-Amy3 gene from rice involves both transcriptional repression and activation in response to the presence or absence of a carbon source. These positive and negative regulations involve the action of different trans-acting factors on the same cis-acting regulatory element (Lu et al., 1998; Lu et al., 2002). To further understand how STP1 responds to changes in the carbon supply, the levels of its transcript were analysed in response to fluctuations in sugar availability. For this purpose, 10-day-old plants grown either in sugar starvation (–C) or in the presence of 150 mM Glc or Mtl for 2 days were transferred to media without a carbon source (–C) or with 150 mM Glc or Mtl for 6 h, and the levels of the STP1 transcript were analysed. In agreement with our previous results, the initial level of STP1 transcript \((T0)\) was lower in the plants grown in the presence of Glc than in those grown in its absence. Independently of the initial STP1 transcript level, the addition of Glc resulted in a drastic reduction of the STP1 transcript in the plant (Fig. 1D). In contrast, when the plants were transferred to media without sugar (–C), the STP1 transcript accumulated (Fig. 1D). In addition, in accordance with published results, the expression of the APL3 gene increased in the presence of Glc, albeit at different levels depending on the initial media in which the seedlings were grown prior to the treatment (Fig. 1D). Together, these data demonstrated that Glc regulation of STP1 mRNA is rapid and dynamic. This result also indicates that multiple elements may be involved in the Glc regulation of STP1, a regulation that is similar to that of the rice \( \alpha \)-Amy3 gene.
Sugar regulation of STP1 responds to the level of phosphorylated hexose

Due to the rapid and sensitive response observed in the accumulation of the STP1 transcript in response to fluctuations in the Glc level, we decided to further investigate the mechanism involved in this regulation. Plants have the capacity to sense different sugars and transmit their signals through different pathways that involve specific components and mechanisms (Xiao et al., 2000; Rolland et al., 2006). The use of Glc analogues has been useful for characterizing the requirements for the regulation of specific genes by sugars (Jang and Sheen, 1994). Thus, we analysed the effect of the Glc analogues mannose (Man) and 3-0mG on STP1 expression. In the presence of 150 mM Man, low levels of the STP1 transcript were observed, similar to those found with 150 mM Glc (Fig. 2A). Man is a Glc analogue that is transported into cells and phosphorylated by hexokinase (HXK) but is very slowly metabolized (Jang and Sheen, 1994; Xiao et al., 2000). In contrast, in the presence of 3-0mG, no repression of the STP1 transcript was observed, and the expression level remained comparable to the one found in the carbon deprivation (–C) condition (Fig. 2A). 3-0mG is transported into the cell (Jang and Sheen, 1997; Lalonde et al., 1999; Smeekens, 2000) but is not phosphorylated because it is a very poor substrate for HXK (Cortes et al., 2003). Finally, similarly to Glc, the addition of sucrose (Suc) resulted in very low STP1 levels (Fig. 2A). The response observed for STP1 to these different sugar analogues was the same with lower levels (5 mM) of these sugars (Fig. 2B). Similar responses to these sugar analogues were found for the DIN6 gene, which is also induced by sugar starvation (Baena-Gonzalez et al., 2007). Under the conditions used in this analysis, only a slight reduction in the expression level of the photosynthetic CABI gene was detected (Fig. 2), suggesting that the response of this gene requires a longer treatment time or higher sugar concentrations. Together, these results support the hypothesis that the signal that initiates the regulation of the STP1 transcript requires a phosphorylatable hexose, such as Glc or Man.

Fig. 2. Regulation of STP1 expression by sugars. The expression of STP1, DIN6, and CABI was analysed by a northern blot from the RNA extracted from 12-day-old plants grown for 2 days in sugar-depleted (–C) media and then transferred to 150 mM (A) or 5 mM (B) Glc, Mtl, 3-0mG (3-0-methylglucose), Man (mannose), or Suc (sucrose) for 6 h. Each lane was loaded with 10 µg of total RNA. The RNA from the methylene blue-stained membrane is shown as a loading control. The blots shown are representative of three biologically independent experiments.

An independent HXK1 pathway drives the sugar regulation of STP1

Previous work has demonstrated that HXK1 functions as a primary Glc sensor that initiates a specific sugar signalling pathway; this pathway then induces or represses the expression of many genes in response to phosphorylated sugars (Moore et al., 2003; Price et al., 2004; Li et al., 2006). Additional components of this signalling pathway include the ABI4 and ABI5 transcription factors (Arenas-Huertero et al., 2000). To determine whether any of these components are required for STP1 sugar regulation, we evaluated the STP1 transcript level in response to Glc in the HXK1 (gin2), abi4-1, and abi5 mutants. As shown in Fig. 3, the level of the STP1 transcript in all three mutants decreased upon Glc addition, similarly to wild-type plants. No decrease in the transcript level was observed in the absence of sugar or in the corresponding Mtl isosmotic control (Fig. 3). In this analysis, we observed that the STP1 transcript level in the abi5 mutant prior to sugar treatment (T0) was lower than that of the wild type and the other mutants (Fig. 3). This result suggests that ABI5, independently of its role in sugar regulation, is required to maintain normal levels of the STP1 transcript. However, these results demonstrated that neither HXK1 nor the transcription factors ABI4 or ABI5 participate in the sugar regulation of the STP1 transcript.

In addition to HXK1, other factors have been shown to play important roles in plant sugar responses, including SnRK1 kinase and a heterotrimeric G protein (Baena-Gonzalez and Sheen, 2008; Urano et al., 2013). To analyse the possible role of these factors in the regulation of STP1 by sugars, we measured the expression of STP1 in the corresponding mutants. In the case of SnRK1 kinase, we evaluated knockout mutants for the two catalytic subunits, kin10 and kin11. Although these catalytic subunits are known to be partially redundant, analysis of a double mutant was not possible due to its lethality (Baena-Gonzalez and Sheen, 2008). In the case of the G protein, we evaluated two independent null mutant alleles of the RGS1 factor (rgs1 and rgs1-2), a protein that modulates G-protein signalling and that has been reported to be an important component for HXK-independent sugar signalling responses (Chen et al., 2003). As shown in Fig. 4A,

Fig. 3. STP1 regulation by Glc is mediated by an HXK1-independent signalling pathway. STP1 expression was analysed in wild-type plants and in the sugar signalling mutants gin2, abi4, and abi5. Samples were obtained from 12-day-old plants grown for 2 days in sugar starvation (–C) conditions and then transferred to –C, 150 mM Glc, or Mtl media for 6 h. Ten micrograms of total RNA was used for northern analysis and was hybridized with the STP1 probe. An initial control, which was measured before the treatments (T0), is included. The rRNA from the methylene blue-stained membranes is shown as a loading control. The membrane shown is representative of two biologically independent experiments.
no difference in the regulation of \textit{STPI} by Glc was found in the \textit{kin10} or \textit{kin11} mutants compared to that in the wild-type Col-0 plants. However, due to the partial redundancy of these subunits, the participation of SnRK1 in the regulation of \textit{STPI} in response to Glc cannot be totally excluded. To further address the function of the SnRK1 complex in the Glc regulation of the \textit{STPI}, two independent lines that overexpress the KIN10 catalytic subunit (\textit{KIN10-OX}) were analysed (Baena-Gonzalez and Sheen, 2008). We hypothesized that if the SnRK1 kinase has any role in the sugar repression of the \textit{STPI} gene, this response should be exacerbated in the \textit{KIN10} gain-of-function lines. Because the level of the \textit{STPI} transcript in the wild-type plants remain unaltered for the first 15 min after the addition of 5 mM Glc (Fig. 1C), we analysed the \textit{STPI} level in two overexpressing lines, \textit{KIN10-OX5.7} and \textit{KIN10-OX6.5}, under these conditions. However, we did not detect any difference in the \textit{STPI} expression level between the overexpressing lines and the wild-type plants 15 min after Glc addition (data not shown). We also analysed \textit{STPI} levels after exposure to Glc for a longer time (30 min). In this case, we detected a slight increase in the repression level in the overexpressing seedlings (Fig. 4B). Densitometric analysis of the \textit{STPI} signal from independent biological experiments showed that repression of \textit{STPI} expression in the \textit{KIN10-OX} lines was 31%, compared to 22% in the wild-type plants. Finally, no difference was detected in the \textit{STPI} sugar regulation in the two mutant alleles of the \textit{RGS1} gene in comparison to that in the wild-type plants (Fig. 4C). These results suggest that none of the factors analysed here play a major role in the regulation of \textit{STPI} by sugars.

The regulation of \textit{STPI} by sugars depends on the DNA elements located in the upstream region of this gene

Transcription plays a major role in the sugar regulation of many genes (Price \textit{et al.}, 2004; Bläsing \textit{et al.}, 2005). In various cases, this regulation depends on the presence of one or more \textit{cis}-acting elements in the promoter region of the sugar-regulated genes (Chen \textit{et al.}, 2006; Li \textit{et al.}, 2006). However, post-transcriptional regulatory mechanisms are also involved in the regulation by sugars (Rolland \textit{et al.}, 2006; Hummel \textit{et al.}, 2009). To characterize the molecular mechanism of the regulation by Glc of the \textit{STPI} gene, a 2.4 kb fragment upstream from the ATG, which includes the upstream regulatory region and the 5’ UTR, was fused to the \textbeta-glucuronidase (GUS) reporter and introduced into \textit{A. thaliana} plants (Fig. 5). Three independent transgenic lines (L1-2, L3-6, and L4-5) were selected, and the presence of the transgene was confirmed by PCR (data not shown). Homozygous plants from each line were selected.

GUS expression patterns of three independent transgenic lines were analysed in 12-day-old seedlings. GUS staining was prominently detected in leaves but it was also present in the vascular system of stems and roots with lower but clearly detectable levels (Fig. 5A). As previously published (Stadler \textit{et al.}, 2003), the site of higher expression at this developmental stage was in leaves (Fig. 5A). A detailed analysis of this organ revealed that \textit{STPI} expression was particular strong in trichomes, including the base of the stalk and the cells around them (Fig. 5D), and in stomata (Fig. 5G). The expression in the guard cells was not homogenous with a more intense GUS activity at the membrane near the stomatal pore (Fig. 5G). GUS staining was also present in the mesophyll cells of the leaves at lower levels (Fig. 5J). In contrast to published data (Stadler \textit{et al.}, 2003), we could not detect differences in the GUS expression between the adaxial or abaxial surfaces of the leaves. However, such a difference might exist but be masked by the diffusion of the GUS marker.

Our previous northern analysis demonstrated that the \textit{STPI} transcript practically disappears after 1 h of Glc addition (Fig. 1B), thus the activity of GUS was followed in the transgenic plants after the addition of 150 mM Glc. In contrast to the RNA analyses noticeable differences in GUS accumulation in the Glc-treated plants were not observed prior to 12 h of Glc exposure (data not shown). After 12 h of Glc treatment a reduction in the GUS activity was observed in all the transgenic lines (Fig. 5B and E). This response is specific for Glc as it is not observed with isosmotic concentrations of Mtl (Fig. 5C and F), which display an indistinguishable GUS level compared to the one observed without the carbon source (Fig. 5A). The decrease in GUS expression in response to the presence of Glc was most prominent in the stomata and...
levels of the GUS transcript were detected in the transgenic plants that were transferred to media without sugar (−); this high expression was independent of the initial growing media prior to the transfer (with or without sugar). In contrast, the GUS transcript was barely detectable when these plants were transferred to media containing 150 mM Glc (Fig. 6). This regulation was very similar to the one observed for the STP1 endogenous transcript (Figs 1C and 6). From this analysis, we concluded that the cis-acting elements responsible for Glc regulation of the STP1 gene are contained in the 2.4 kb fragment, at least under the conditions analysed.

Delimiting the cis-acting regions of the STP1 promoter that respond to Glc.

In order to narrow down the specific elements involved in the sugar regulation of STP1, three consecutive deletions of the original 2.4 kb upstream fragment were generated and fused to GUS; each containing 1.3 kb, 1 kb, and 0.5 kb from the original fragment (Fig. 7). Independent transgenic lines were selected from each deletion based on their resistance to kanamycin, and the deletion size was corroborated by PCR (data not shown). Homozygous plants from a representative line were selected for each deletion, and the expression levels of STP1 and GUS were analysed after sugar treatments. Treatments were conducted using 10-day-old plants starved of a carbon source for two days, after which the plants were transferred to different media (−C, Glc, or Mtl) for 6 h. The expression level of the GUS transgene was compared to the level prior to the transfer (T0) in each deletion. We observed that the basal GUS transcript level (T0) was considerably lower in all deletions than the level observed in the 2.4 kb fragment (Fig. 7). This result is particularly evident in the pSTP1-1.3::GUS and pSTP1-0.5::GUS constructs, indicating that important elements required for normal STP1 expression level are localized between the deleted sequences in each case. However, independently of the basal transcript level (T0), the presence of 150 mM Glc repressed the GUS transcript level in the pSTP1-1.3::GUS deletion; the extent of repression was similar to the one observed with the initial pSTP1-2.4::GUS construct (Fig. 7). In contrast, minor differences, if
any, in the level of GUS transcript were observed in the two additional deletions (pSTP1-1::GUS, and pSTP1-0.5::GUS) in the presence of Glc (Fig. 7). Densitometric analysis from independent lines and independent experiments demonstrated that the presence of Glc results in a 98% reduction in the pSTP1-1.3::GUS lines (P = 0.001) compared with the T0 control plants; only a 30% reduction was detected for the pSTP1-1::GUS lines (P = 0.004) (Fig. 7). The efficiency of the treatments was corroborated by the response of the endogenous STP1 transcript (Fig. 7). Together, these results demonstrate that Glc regulates STP1 expression at the transcriptional level and that repression by sugar depends on cis-acting sequences contained within a 309 bp fragment, which is localized between 1.3 and 1 kb upstream of the STP1 translational initiation codon (Fig. 7).

In silico analysis of putative cis Glc-responsive elements in the 309 bp STP1 promoter fragment.

Previous studies have identified cis-regulatory elements for independent genes that participate in repression by sugars (Hwang et al., 1998; Morita et al., 1998; Toyofuku et al., 1998; Tatematsu et al., 2005). Thus, we performed an in silico analysis of the 309 bp fragment and searched for motifs that are known to be involved in the repression by sugars. This analysis was performed using the PLACE database (http://www.dna.affrc.go.jp/PLACE/) and by also considering additional cis elements that were found to be overrepresented in the regulatory regions of sugar-repressed genes (Li et al., 2006; Baena-Gonzalez et al., 2007). This analysis revealed 17 potential elements in the 309 bp fragment (Fig. 8 and Table 1) that belonged to eight different sugar motifs. One of the most interesting elements found in this region corresponds to the TATCCAOSAMY motif. This motif occurs twice in the 309 bp STP1 fragment (at –1134 and –1155 from ATG); the two instances are separated by 15 bp (Fig. 7). The TATCCAOSAMY motif was originally found in the 5’ upstream regulatory region of the α-Amy3D gene from rice and has been demonstrated to be essential for the regulation of this gene by sugars (Lu et al., 1998; Lu et al., 2002). Part of the TATCCAOSAMY motif (TATCC) overlaps with two other elements (MYBST1 and I-BOX) in the complementary strand and in reverse orientation (Table 1). The I-BOX is found four times within this sequence (Fig. 8), but only two of these instances overlap with the TATCCAOSAMY motif. An additional element in this region was the CGACGOSAMY3 motif (Hwang et al., 1998), which localized at –1104 in the STP1 promoter (Fig. 8). This motif was also originally described in the promoter of the α-Amy3D gene and is required for the Glc repression of this gene (Hwang et al., 1998). In addition, we identified seven sequences with homology to three elements that are overrepresented in the sugar-repressed genes in the microarray data reported by Li et al. (2006). Four of these sequences share homology with the GATTA motif, two with the EVENINGAT core element and one with the CATCC motif (Fig. 8 and Table 1).

Similar to STP1, the expression of the DIN6 gene is strongly repressed by the presence of sugars and activated during sugar starvation (Baena-Gonzalez et al., 2007). Thus, we decided to compare 309 bp of the STP1 fragment with the upstream sequence of DIN6 gene. This analysis revealed only two motifs that were shared between these sequences: one of them is the TATCCAOSAMY motif, and the other is a sequence related to a G-box (ACGTG) that are known to be involved in the repression by sugars. This motif was also originally described in the 5’ upstream regulatory region of the α-Amy3D gene from rice and has been demonstrated to be essential for the regulation of this gene by sugars (Lu et al., 1998; Lu et al., 2002). Part of the TATCCAOSAMY motif (TATCC) overlaps with two other elements (MYBST1 and I-BOX) in the complementary strand and in reverse orientation (Table 1). The I-BOX is found four times within this sequence (Fig. 8), but only two of these instances overlap with the TATCCAOSAMY motif. An additional element in this region was the CGACGOSAMY3 motif (Hwang et al., 1998), which localized at –1104 in the STP1 promoter (Fig. 8). This motif was also originally described in the promoter of the α-Amy3D gene and is required for the Glc repression of this gene (Hwang et al., 1998). In addition, we identified seven sequences with homology to three elements that are overrepresented in the sugar-repressed genes in the microarray data reported by Li et al. (2006). Four of these sequences share homology with the GATTA motif, two with the EVENINGAT core element and one with the CATCC motif (Fig. 8 and Table 1).

Finally, several members of the STP family are also repressed by sugars (Price et al., 2004). Thus, we also searched for common motifs between STP1 and three other known sugar-regulated members. For this analysis, the complete upstream intergenic regions of the STP4 (At3g19930), STP13 (At5g26340), and STP14 (At1g77210) genes were compared against the 309 bp fragment of the STP1 promoter. Six out of the eight different motifs previously identified in STP1 were also present at least once in the control regions of the other STP genes (Table 1). Interestingly, the CGACG and the TATCCAOSAMY motifs were found in STP4 and STP13 genes, but not in the STP14 gene.
Discussion

Sugars act as key regulators of gene expression by inducing or repressing the transcription of many genes (Koch, 1996). Many studies have contributed to understanding the mechanisms by which sugars regulate gene expression (Rolland and Sheen, 2005; Eveland and Jackson, 2012). Initial forward genetics studies were valuable for demonstrating the complexity of sugar signalling and provide evidence for the existence of multiple signalling pathways. However, genomics and system biology analyses have been crucial for demonstrating the effect of sugar availability on expression throughout the entire genome (Price et al., 2004; Villadsen and Smith, 2004; Gutierrez et al., 2007; Osuna et al., 2007). The STP family is one of the gene families that has repeatedly been detected in genomic analyses as highly responsive to sugars (Price et al., 2004; Villadsen and Smith, 2004). This family includes genes that encode low- and high-affinity monosaccharide transporters (Stadler et al., 2003; Büttner, 2010; Slewinski, 2011). Compared to other members of the family, STP1 is a high-affinity H⁺/sugar cotransporter with the highest and broadest expression in A. thaliana (Büttner, 2010). Our data corroborated the findings that the expression of STP1 is rapidly modulated by minor fluctuations in sugars levels (5 mM Glc). This response contrasts with the response of other sugar-repressed genes, such as several photosynthetic genes, that require higher sugar levels and a longer time to affect the level of their transcripts (Acevedo-Hernandez et al., 2005).

In addition to STP1, other members of the STP family have also been shown to be regulated by sugars; however, whether these involve common mechanisms is unknown. Previous work demonstrated that STP1 expression is induced by darkness and repressed by light in guard cells. This regulation has been suggested to be important for the import of carbon to these cells, particularly during dark periods (Stadler et al., 2003). Our data with transgenic lines containing the pSTP1:GUS fusion corroborated the view that one of the sites with major levels of GUS accumulation corresponds to the stomatal guard cells. Interestingly, this expression is notably decreased with exposure to Glc. Thus it is likely that at least part of regulation previously observed by light is linked to the sugar fluctuations in these cells during dark periods more than a direct downregulation by light. Since guard cells depend on sugar import to maintain their metabolism as they are unable to perform photosynthesis, it is likely that during dark periods the levels of phosphorylatable hexoses become very low and in consequence the expression of the STP1 gene gets induced. Previous work has

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Table 1. Known cis-acting elements involved in sugar repression in the 309 bp fragment from the STP1 promoter

| Element              | Sequence       | Reference                        |
|----------------------|----------------|----------------------------------|
| CGACGOSAMY3          | CGAGG          | Hwang et al., 1998               |
| TATCCAOAMY           | TATCCA         | Lu et al., 1998                  |
| SREATMSD             | TATCC          | Tanematsu et al., 2005           |
| TATCCAYMOTIFOSRAMY3D | TATCCAY        | Toyofuku et al., 1998            |
| MYBST1               | GQATA          | Baranowskij et al., 1994         |
| I-BOX core           | GATAA          | Han et al., 2000; Li et al., 2006|
| EVENINGAT core       | ATATCT         | Harmer et al., 2000; Li et al., 2006|
| CATCC                | CATCC          | Li et al., 2006                  |
| GATTA                | GATTA          | Li et al., 2006                  |
| G-box related        | ACGTG          | Lu et al., 1998; Baena-Gonzalez et al., 2007|

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Fig. 8. Putative sugar regulatory motifs in the 309 bp region of the STP1 promoter. The numbers indicate the position of the last base in each motif and refer to the translation initiation site of STP1. The overlapping elements are underlined. The arrowheads indicate elements found in reverse orientation and (–) in the complementary strand.
found an increase in $STPI$ mRNA at the onset of the dark period. We could speculate that the decrease in sugar import as a result of the lack of photosynthetic activity, together with the start of starch breakdown that will supply carbon skeletons during the next hour, may mean that the actual intracellular phosphorylatable hexose levels are very low. The expression of a high-affinity sugar transporter such as $STPI$ under these conditions is possibly important for transporting available external hexoses. Although additional experiments will be required to clarify these aspects, the sensitive and rapid response observed here for $STPI$ expression is very well suited to ensuring proper sugar influx in response to minor fluctuations in sugar availability in guard cells as well as in other plant tissues. The other sites where high GUS expression was detected are the trichomes. However, the physiological reason for the requirement of this transporter in this type of specialized structure is less obvious and will require future analyses.

Considering the mechanisms, we believe that the rapid response of the $STPI$ transcript to fluctuations in sugar levels suggests that some of the elements involved in the perception of the Glc signal should be present prior to the stimulus. This possibility agrees with the observation that the Glc repression of other $STP$ genes ($STP14$ and $STP4$) is normal in the presence of the translational inhibitor cycloheximide (Price et al., 2004). Surprisingly, that study also found that the repression level of the $STPI$ transcript appears to be less severe in the presence of cycloheximide. Thus, it is possible that the de novo synthesis of some of the trans-acting factors is required either to achieve full repression or to sustain this response (Price et al., 2004). The present analysis also reveals that the half-life of the $STPI$ transcript is apparently not very long; thus, the repression of the transcription level is reflected in the total transcript level within minutes of Glc addition.

Our analyses of $STPI$ expression using different Glc analogues demonstrated that the signal that induces the repression of this gene is a phosphorylatable sugar. These data agree with previous reports that found that the non- or poorly phosphorylatable Glc analogues, such as 3-OmG and 6-deoxyglucose, did not change $STPI$ expression (Cortes et al., 2003; Villadsen and Smith, 2004). Interestingly, our data also demonstrated that the sugar signal that modulates the repression of the $STPI$ gene is independent of the HXK1 sensor. Therefore, a primary sensor different from HXK1 must perceive the phosphorylated sugars that initiate $STPI$ regulation. In spite of the important efforts of many groups, still almost nothing is known about alternative receptors for sugar perception with the exception of the regulator of G protein (RGS1). RGS1 has been suggested to bind sugars and attenuate the cell division of the apical root meristem through its interaction with a heterotrimeric G protein independently of HXK1 (Chen et al., 2003; Chen, 2008). However, in this work, we demonstrated that RGS1 does not appear to play a major role in the sugar regulation of $STPI$ because the repression of $STPI$ by sugars is very similar to the repression in wild-type plants in the absence of this regulator.

The genome of most plants encodes various HXK-related genes in addition to HXK1: five in the case of $A. thaliana$ and ten in rice (Granot et al., 2013). Although some of these HXK genes have clear enzymatic activity (type A and B), others apparently lack such activity (HKL) and have been suggested to have regulatory functions (Xiao et al., 2000; Rolland et al., 2006; Karve et al., 2008; Granot et al., 2013). In fact, recent work provided evidence that different HXK genes have signalling roles in different plants. For example, several HXK-type B genes from potato and rice were able to complement the Glc sensitivity of the gin2 mutant (Veramendi et al., 2002; Cho et al., 2009; Karve et al., 2010). In addition, a signalling role was observed for some HKL-type genes in $A. thaliana$ and Physcomitrella (Thelander et al., 2005; Zhang et al., 2010; Karve et al., 2012). Whether any of the additional HXK genes (A, B, or HKL) have a role in the sugar regulation of $STPI$ remains for future analysis.

Other players that have been shown to participate in sugar signalling are the SnRK1 and TOR kinases (Baena-Gonzalez and Sheen, 2008; Xiong et al., 2013). SnRK1 kinase is highly conserved throughout the evolution of different organisms, including plants, and has been demonstrated to be crucial for energy homeostasis, such as carbon availability (Hardie et al., 1998; Baena-Gonzalez, 2010). Importantly for the present study, alterations in $STPI$ expression were reported in a microarray analysis from transiently overexpressing KIN10 protoplasts (Baena-Gonzalez et al., 2007). $A. thaliana$ contains two SnRK1 catalytic subunits (KIN10 and KIN11) that are partially redundant (Baena-Gonzalez et al., 2007). However, it was not possible to analyse the double mutant due to its lethality; therefore, in this work, we explored the role of this kinase in the regulation of the $STPI$ gene using the single kin10 and kin11 mutants (Polge and Thomas, 2007) as well as transgenic lines that overexpress KIN10. KIN10 has been reported to have the most notable activity of the two catalytic subunits (Jossier et al., 2009). In this analysis, we did not observe major differences in the response of $STPI$ to sugars in any of the various analysed mutants and lines. Thus, although the involvement of this kinase in the regulation of $STPI$ cannot be completely ruled out, the only difference we observed is a slight reduction in the level of the $STPI$ transcript in the overexpressing KIN10 lines. Our data indicate that the participation of SnRK1, if any, in the regulation of $STPI$ is minor.

None of the factors analysed so far play a major role in the regulation of $STPI$, suggesting the participation of novel factors in the regulation of this gene. Potential additional candidates include factors whose mutants display alterations in $STPI$ expression. For example, in comparison to the wild-type plants, the sweetie mutant displays an upregulation of the $STPI$ gene (Veyres et al., 2008). SWEETIE encodes a novel protein of unknown function and is implicated in various processes, including sugar perception, senescence, ethylene biosynthesis, and abiotic stresses (Veyres et al., 2008; Büttner, 2010). Misregulation of the $STPI$ gene by sugars was also reported in hsr (high sugar-response) mutants. For several genes, these mutants displayed sugar hypersensitivity, and the elements that are affected in these mutants are good candidates for involvement in $STPI$ sugar regulation.
Unfortunately, the identities of the HSR genes are still unknown (Baier et al., 2004).

In this work, we also demonstrated that sugar regulates the STP1 gene at the transcriptional level, and this regulation is similar to that of the sugar-regulated genes DIN6/ASN1 and \(\alpha\)-Amy3, whose expression is also induced by sugar starvation and is repressed in its presence (Lam et al., 1998; Lu et al., 2002; Baena-Gonzalez et al., 2007). Similarly to the DIN6 and DIN1 genes, the regulation by Glc of STP1 is independent of the HXK1 pathway (Baena-Gonzalez et al., 2007). These similarities support a common mechanism for the regulation of these genes by sugars. In the present analysis, we were able to delimit the cis-acting elements required for the STP1 sugar repression within 309 bp. Our in silico analyses showed the cis-acting elements that are common to the STP1 309 bp sequence and the \(\alpha\)-Amy3 and DIN6 promoters, including the TATCCA and the G boxes (Lu et al., 2002; Baena-Gonzalez et al., 2007).

The TATCCA element (TATCCAOAMY) was originally identified as the binding site of one MYB-type transcription factor (OsMYBS2) that is essential for the sugar regulation of the \(\alpha\)-Amy3 gene in rice (Lu et al., 2002). Moreover, the arrangement of these elements in the STP1 promoter (in tandem and separated by 15 bp) is similar to that in the \(\alpha\)-Amy3 gene (Lu et al., 1998). Thus, this sequence is an interesting candidate for involvement in the regulation of the STP1 gene by sugars. MYB transcription factors are members of a large gene family in plants with more than a hundred members in *A. thaliana* (Dubos et al., 2010). Two MYB genes in *A. thaliana* (At5g47390 and At5g61620) are the closest to the rice MYBS2 factor based just on protein sequence identity. However, the role of this putative orthologue requires further study.

The STP14 gene does contain five TATCCA elements in the 5’ UTR that is shared with both STP1 and DIN6. However, neither the TATCCAOAMY nor the CGACGOSAMY3 motifs are present in the upstream sequence of the STP14 gene; this gene and STP1 are among the most sugar-repressed genes of the STP family (Price et al., 2004). Thus, the contribution of any of these elements to the control by Glc of the STP genes must be determined in the future.

Finally, a low but reproducible increase in the STP1 transcript at midday was reported and linked to a circadian regulation of this gene (Harmer et al., 2000; Stadler et al., 2003). This is an interesting aspect taking into account that one of the motifs present in the region responsible for sugar regulation includes the EVENINGAT element present in genes regulated by the circadian clock (Harmer and Kay, 2000). This element was also found overrepresented in the sugar-repressed genes in a microarray data reported by Li et al. (2006). It is possible that the expression of the STP1 gene, like many other genes, might be subjected to multiple regulatory mechanisms, in addition to sugars. However, recent evidence supports the view that the levels of sugars directly influence the circadian regulation of many genes (Haydon et al., 2013), supporting possible crosstalk between these regulatory mechanisms. Although that there is still not a direct probe to show that the EVENINGAT element might be directly involved in sugar regulation, this aspect is an interesting possibility that requires further exploration in the future.

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