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# Differential Effects of Carbohydrates on Arabidopsis Pollen Germination

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**Pollen germination as a crucial process in plant development strongly depends on the accessibility of carbon as energy source. Carbohydrates, however, function not only as a primary energy source, but also as important signaling components. In a comprehensive study, we analyzed various aspects of the impact of 32 different sugars on in vitro germination of Arabidopsis pollen comprising about 150 variations of individual sugars and combinations. Twenty-six structurally different mono-, di- and oligosaccharides, and sugar analogs were initially tested for their ability to support pollen germination. Whereas several di- and oligosaccharides supported pollen germination, hexoses such as glucose, fructose and mannose did not support and even considerably inhibited pollen germination when added to germination-supporting medium. Complementary experiments using glucose analogs with varying functional features, the hexokinase inhibitor mannoheptulose and the glucose-insensitive hexokinase-deficient Arabidopsis mutant *gin2-1* suggested that mannose- and glucose-mediated inhibition of sucrose-supported pollen germination depends partially on hexokinase signaling. The results suggest that, in addition to their role as energy source, sugars act as signaling molecules differentially regulating the complex process of pollen germination depending on their structural properties. Thus, a sugar-dependent multilayer regulation of Arabidopsis pollen germination is supported, which makes this approach a valuable experimental system for future studies addressing sugar sensing and signaling.**

**Keywords:** *Arabidopsis thaliana* • Carbohydrates • Metabolic regulation • Pollen germination • Signaling • Structure–function relationship.

**Abbreviations:** Col, Columbia; DAF III, α-D-fructofuranose-β-D-fructofuranose-1,2′:2,3′-dianhydride; DOG, deoxyglucose; FBP, FRUCTOSE-1,6-BISPHOSPHATASE; 1F-Fru, 1-fluoro-1-deoxy-D-fructose; FINS1, FRUCTOSE INSENSITIVE1; FRK, fructokinase; Fru, fructose; Gal, galactose; *gin*, glucose insensitive; Glc, glucose; GPM, glucopyranosyl-mannitol; GPS, glucopyranosyl-sorbitol; HXK, hexokinase; L*er*, Landsberg *erecta*; Man, mannose; ManOH, mannitol; Mhl, mannoheptulose; OMG, *O*-methyl-Glc; Sor, sorbose; SorOH, sorbitol; STP, sugar transport protein; Suc, sucrose; SUT, sucrose transporter; SWEET, sugars will eventually be exported transporter.

## Introduction

As autotrophic organisms, plants assimilate carbon as energy source and for the synthesis of biomass and various chemical compounds. Sugars are the main energy source for the plant's metabolism but they also function as important signaling molecules regulating growth and development (Gibson 2005, Hanson and Smeekens 2009, Smeekens et al. 2010). In this role, different sugar molecules such as sucrose, glucose (Glc) or trehalose-6-phosphate are suggested as signaling components to regulate various plant processes (Ruan 2014) in which they also interact in complex networks with other signaling pathways in plants such as those for inorganic nutrients, light, hormones and different stress factors (Rolland and Sheen 2005, Hanson and Smeekens 2009, Matsoukas 2014). These potential signaling effects thereby strongly depend on the individual sugar, as indicated, for example, by the differential impact of various metabolizable and non-metabolizable sugars on mitogen-activated protein kinase (MAPK) signaling in tomato (Sinha et al. 2002).

Within these signaling networks, it is pivotal for plants to sense the presence and absence of sugars (Hoth et al. 2010, Ruan 2014). One of the sensors playing a central role in sugar metabolism and sugar signaling is the enzyme hexokinase (HXK; Roitsch et al. 1995; Rolland et al. 2006, Granot et al. 2013, Sheen 2014). HXK is able to phosphorylate Glc and, with much lower affinity, also fructose (Fru; Granot 2007). The resulting hexose-6-phosphates can either enter glycolysis or serve as the starting point for producing other metabolites. Dissection of the catalytic properties from the signaling function of the *Arabidopsis thaliana* AtHXK1 by site-directed mutagenesis (Moore et al. 2003) showed that HXK is a moonlighting enzyme as it exhibits a sugar-sensing function (Jang et al. 1997) in addition to its catalytic activity (Moore 2004).

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Exogenous application of different carbohydrates during seed germination of *A. thaliana* revealed a strong influence of sugar composition in this early process of plant life (Jang and Sheen 1994, Gibson 2005, Rognoni et al. 2007). Other processes indispensably connected to sugars as energy source are pollen germination and pollen tube growth (Reinders 2016, Goetz et al. 2017). The contact with a receptive stigma leads the pollen grain to rehydrate and, after polarization of the vegetative cell, a pollen tube protrudes out of an aperture (Edlund et al. 2004). During this strictly polar process, growth rates of up to 1 cm $h^{-1}$ can be achieved as reported for maize (Barnabas and Fridvalszky 1984) and, already 7 h after pollination, the ovules of *A. thaliana* are fertilized (Faure et al. 2002). This rapid growth is highly energy consuming, but sugars are also discussed to be involved in pollen tube guidance, since Reger et al. (1992) proved that a Glc gradient influences pollen tube growth in pearl millet, and the glycosylation gradient of hydroxyproline-rich arabinogalactan proteins increases from the stigma to the ovary in the styles of *Nicotiana tabacum* (Wu et al. 1995).

The aim of this study was to investigate the effect of various sugars (Supplementary Fig. S1) comprising different naturally occurring mono-, di- and oligosaccharides as well as commercially available and newly synthesized sugar analogs, during pollen germination. Pollen can easily be cultivated in vitro where pollen germination and pollen tube growth will not be influenced by other signals deriving from surrounding tissues. We established in vitro pollen germination as an experimental system to study the effects of exogenously applied sugars. We show that (i) *A. thaliana* pollen germination is differentially regulated by exogenously available carbohydrates; (ii) that sucrose (Suc) strongly supports pollen germination while hexoses inhibit pollen germination; and (iii) that Glc-, and especially mannose (Man)-dependent inhibition of pollen germination involves a HXK-mediated signaling pathway.

## Results

### Different support of in vitro pollen germination by mono-, di- and oligosaccharides

Established protocols for germination of *A. thaliana* pollen include Suc as sole carbon source (Stadler et al. 1999, Boavida and McCormick 2007). In vitro assays were performed to investigate the influence of different carbohydrates on pollen germination, which comprise a selection of sugars covering various structural properties such as the different basic structure of the monosaccharides or the type of glycosidic bonds in oligosaccharide molecules (Supplementary Fig. S1). These structural properties determine functional differences such as the ability to be phosphorylated or metabolized. In total, 26 individual carbohydrates were tested for their ability to support pollen germination at a concentration of 440 mM as used for Suc in the standard medium (**Table 1**), including the five hexoses Fru, Glc, galactose (Gal), Man, sorbose (Sor) and two sugar alcohols [mannitol (ManOH) and sorbitol (SorOH)]. The 16 disaccharides tested comprise Suc, the two Suc isomers turanose and palatinose, the two sugar alcohol-containing glucopyranosyl-mannitol (GPM) and glucopyranosyl-sorbitol (GPS), the two chloride-substituted Suc analogs sucralose and dichlorosucrose (replacement of OH groups by Cl groups) and the Fru disaccharide α-D-fructofuranose-β-D-fructofuranose 1,2′:2,3′-dianhydride (DAF III). In addition, the three sucrose-containing oligosaccharides melezitose, stachyose and raffinose were tested.

Most monosaccharides as the exclusive carbon source in the medium did not support pollen germination. Only Glc supported pollen germination to a very limited extent (2.8%), whereas all the other monosaccharides, i.e. the hexoses Fru, Gal, Man, Sor and the monosaccharide alditols SorOH and ManOH did not support pollen germination at all (**Table 1**). The tested disaccharides differed considerably in their ability to support pollen germination. Sucrose and cellobiose (48.9 and 50.3% pollen germination, respectively) as well as maltose (30.0% pollen germination) strongly supported pollen germination, whereas addition of lactose, leucrose, melibiose and trehalose to the medium only resulted in a very limited pollen germination (<10%). The synthetic DAF III, lactulose and disaccharide alditols (GPM and GPS) and the two non-plant-occurring Suc isomers did not support pollen germination at all, similarly to media containing sucralose and dichlorosucrose. In contrast, the trisaccharides melezitose and raffinose as well as the tetrasaccharide stachyose resulted in high pollen germination rates comparable with Suc (**Table 1**). These results can be rationalized assuming that the efficiency in the uptake of the di- and oligosaccharides is the rate-limiting step determining their pollen germination-supportive capabilities. Sucrose, sucrose oligosaccharides and the α- and β-(1→4)-linked glucodisaccharides maltose and cellobiose are known to be readily internalized through sucrose transporters (Sivitz et al. 2007) and are subsequently hydrolyzed by the action of invertase or α- and β-glucosidases (Roitsch and Gonzalez 2004). Only glucosides were transported by type I SUCs from dicots and type II SUCs from monocots (Sivitz et al. 2007). Notably, the two pollen germination-promoting mono- and digalactosides of sucrose (raffinose and stachyose) and melezitose are substrates of invertases (Hasegawa and Smolensky 1970, Goetz and Roitsch 1999). The affinity of sucrose transporters for dipyranosidic disaccharides having galactopyranosyl (lactose, melibiose) or α-glucopyranosyl units with other types of glycosidic linkages (leucrose, trehalose) is probably much lower. Since they nevertheless support pollen germination, though to a lesser extent, enzymatic hydrolysis into their constitutive monosaccharides to provide a carbon source seems not to be a constraint. The lack of capabilities to support pollen germination of the non-metabolizable disaccharide lactulose as well as of the disaccharides turanose, palatinose and the two glucopyranosyl alditols, which would be substrates of α-galactosidase or α-glucosidase, can then be ascribed to their lack of affinity for the sucrose transporters (Sivitz et al. 2007). It is worth noting that in the three disaccharides showing no pollen germination-supportive activity, the reducing Fru unit is to a large extent (lactulose, turanose) or exclusively (palatinose) in the furanose form, whereas the alditol moiety in the glucosyl alditols is linear. It thus seems reasonable to hypothesize that the presence of a

**Table 1** Influence of different carbohydrates (440 mM) on pollen germination (PG)

| | Sugar | Structure | PG (%) |
|---|---|---|---|
| | ($H_2O$) | | 0*** |
| Monosaccharides | Fructose | | 0*** |
| | Galactose | | 0*** |
| | Glucose | | 2.8 (± 1.0)*** |
| | Mannitol | | 0*** |
| | Mannose | | 0*** |
| | Sorbitol | | 0*** |
| | Sorbose | | 0*** |
| Disaccharides | Cellobiose | D-Glc-β-(1→4)-D-Glc | 50.3 (± 2.2) |
| | DAF III | α-D-Fru β-D-Fru 1,2′:2,3′-dianhydride | 0*** |
| | Dichlorosucrose | 6-Chloro-6-deoxy-D-Glc-α-(1→2)-6-chloro-6-deoxy-D-β-Fru | 0*** |
| | GPM | (α-D-Glc-(1→1)-D-mannitol) | 0*** |
| | GPS | α-D-Glc-(1→6)-D-sorbitol | 0*** |
| | Lactose | D-Gal-β-(1→4)-β-D-Glc | 7.1 (± 0.8)*** |
| | Lactulose | D-Gal-B-(1→4)-Fru | 0*** |
| | Leucrose | α-D-Glc-(1→5)-Fru | 2.6 (± 0.2)*** |
| | Maltitol | α-D-Glc-(1→4)-D-sorbitol | 0*** |
| | Maltose | α-D-Glc-(1→4)-α-D-Glc | 30.0 (± 3.7)*** |
| | Melibiose | α-D-Gal-(1→6)-D-Glc | 9.8 (± 2.0)*** |
| | Palatinose | α-D-Glc-(1→6)-D-Fru | 0*** |
| | Sucralose | 1,6-Dichloro-1,6-dideoxy-β-D-Fru-4-chloro-4-deoxy-α-D-Gal | 0*** |
| | Sucrose | (D-Glc-α-(1→2)-D-β-Fru) | 48.9 (± 1.0) |
| | Trehalose | (α-D-Glc-(1→1)-α-D-Glc) | 4.9 (± 1.6)*** |
| | Turanose | (α-D-Glc-(1→3)-Fru) | 0*** |
| Oligosaccharides | Melezitose | α-D-Glc-(1→3)-β-D-Fru-(2→1)-α-D-Glc | 42.8 (± 1.7)* |
| | Raffinose | α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru | 47.4 (± 1.8) |
| | Stachyose | α-D-Gal-(1→6)-α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru | 55.4 (± 0.4) |

Values represent the means (± SEM).
* and *** indicate significantly different support of PG compared with standard Suc-containing medium at the 0.05 and 0.001 levels of confidence, respectively.
DAF III, α-D-fructofuranose-β-D-fructofuranose-1,2′:2,3′-dianhydride; Fru, fructose; Gal, galactose; Glc, glucose; GPM, glucopyranosyl-mannitol; GPS, glucopyranosyl-sorbitol.

sucrose portion or a dipyranosidic moiety (preferentially a glucodisaccharide with a 1→4 linkage) is essential for efficient internalization and subsequent supportive effect in pollen germination. The inability of DAF III and the synthetic halosucroses to support pollen germination is in agreement with their enzymatic stability, meaning that even if they might be transported they will not provide the necessary energy, and can be considered as negative controls in this assay.

## Hexoses inhibit sucrose-mediated pollen germination

None of the monosaccharides (except a slight effect of Glc) and only specific disaccharides supported pollen germination when added alone (440 mM) to the medium (**Table 1**). To determine whether this reflects simply an inability of certain sugars to support pollen germination as sole carbon source or a specific inhibitory effect, all tested sugars were added in two lower concentrations (6 and 60 mM) to standard medium containing 440 mM Suc. Such a medium proved very suitable for in vitro germination of *A. thaliana* pollen (Hülskamp et al. 1995; **Table 1**) and, in addition, Suc is the major transport form for carbohydrates in higher plants. The assumption was that, particularly at a concentration of 6 mM, a decrease in pollen germination can be attributed to specific inhibitory rather than competitive effects.

The addition of Fru, Glc and especially Man at the very low concentration of 6 mM to standard Suc-containing medium caused a significant inhibition of pollen germination, in comparison with the Suc control (**Fig. 1**). Increasing the concentration of the hexoses to 60 mM resulted in a stronger and significant inhibition of Suc-supported pollen germination; Man, Gal and 1-fluoro-1-deoxy-D-fructose (1F-Fru) even completely inhibited pollen germination. The addition of Sor and the sugar alcohols ManOH and SorOH had no or only very slight inhibitory effects on Suc-supported pollen germination at a concentration of 6 mM, but they significantly inhibited pollen germination at 60 mM (**Fig. 1**). In contrast to the tested hexoses, most of the di- and oligosaccharides exhibited no considerable inhibitory effect on Suc-supported pollen germination at a concentration of 6 mM (**Table 2**), independent of whether they supported pollen germination or not when added alone to the medium (**Table 1**); only addition of 6 mM sucralose and turanose, which themselves do not support pollen germination, resulted in a significant inhibition of Suc-supported pollen germination. At a concentration of 60 mM GPS, palatinose, sucralose and turanose, which all themselves do not support pollen germination, resulted in a distinct inhibition of pollen germination (**Table 2**). Interestingly, the inhibitory effect of turanose was the same for both concentrations while 60 mM sucralose caused a much stronger inhibition than

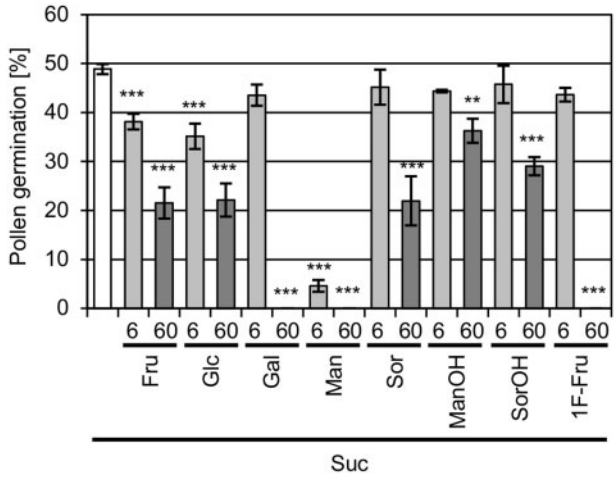


**Fig. 1** Differential inhibitory effect of monosaccharides on sucrose-mediated pollen germination (PG). Suc- (440 mM) containing standard PG medium was supplemented with the indicated monosaccharides at 6 mM (light gray columns) or 60 mM (dark gray columns) concentration. Values (% PG) represent means (± SEM); ** and *** indicate significantly different PG compared with standard Suc-containing medium (white column) at the 0.01 and 0.001 levels of confidence, respectively. Fru, fructose; Gal, galactose; Glc, glucose; Man, mannose; ManOH, mannitol; Sor, sorbose; SorOH, sorbitol; Suc, sucrose; 1F-Fru, 1-fluoro-1-deoxy-D-fructose.

**Table 2** Concentration-dependent influence of di- and oligosaccharides on sucrose-mediated pollen germination (PG)

| | PG (%) | |
|---|---|---|
| **Sugar added** | **6 mM** | **60 mM** |
| – | 48.9 (± 1.0) | |
| Cellobiose | 50.1 (± 0.8) | 48.9 (± 0.6) |
| DAF III | 48.5 (± 3.4) | 46.1 (± 4.0) |
| Dichlorosucrose | 59.7 (± 2.2)** | 51.9 (± 2.7) |
| GPM | 48.2 (± 1.0) | 40.8 (± 2.1) |
| GPS | 45.8 (± 0.6) | 35.1 (± 4.4)** |
| Lactose | 51.0 (± 1.0) | 51.5 (± 1.0) |
| Lactulose | 52.7 (± 0.2) | 48.4 (± 0.8) |
| Leucrose | 48.1 (± 0.0) | 48.0 (± 0.1) |
| Maltitol | 52.5 (± 5.7) | 59.1 (± 2.3)* |
| Maltose | 50.4 (± 1.8) | 45.9 (± 2.4) |
| Melibiose | 44.4 (± 1.1) | 44.0 (± 3.0) |
| Palatinose | 43.8 (± 3.3) | 29.4 (± 4.9)*** |
| Sucralose | 35.9 (± 6.6)** | 20.8 (± 3.3)*** |
| Trehalose | 45.3 (± 1.5) | 48.5 (± 3.7) |
| Turanose | 42.4 (± 3.1)* | 42.3 (± 2.7)* |
| Melezitose | 48.2 (± 2.8) | 52.3 (± 2.0) |
| Raffinose | 48.1 (± 1.0) | 49.6 (± 2.4) |
| Stachyose | 43.5 (± 4.6) | 51.0 (± 1.5) |

Suc (440 mM)-containing standard pollen germination medium was supplemented with the indicated sugars at a concentration of 6 or 60 mM.
Values represent means (± SEM).
*, ** and *** indicate significantly different PG compared with standard Suc-containing medium at the 0.05, 0.01 and 0.001 levels of confidence, respectively.
DAF III, α-D-fructofuranose-β-D-fructofuranose-1,2′:2,3′-dianhydride; GPM, glucopyranosyl-mannitol; GPS, glucopyranosyl-sorbitol.

6 mM Therefore, turanose-based inhibition seems to be independent of competition effects while sucralose causes mixed inhibition, and all other di- and oligosaccharides that inhibited pollen germination only at 60 mM seem rather to compete with Suc as substrate. In general, no correlation between pollen germination rate and pollen tube growth was found as the pollen tube length observed in media containing combinations of two sugars was comparable with tube growth in the standard Suc-containing medium. Only a combination of 440 mM Suc with 60 mM sucralose or dichlorosucrose, respectively, resulted in shorter pollen tubes (Supplementary Fig. S2). However, reducing the concentration of these two chloride-substituted Suc derivatives to 6 mM resulted in pollen tubes not differing in length as compared with tubes grown in Suc alone.

## Inhibition of sucrose-supported pollen germination by glucose and mannose involves a hexokinase-mediated pathway

To study the potential influence of HXK on the hexose-mediated inhibition of Suc-supported pollen germination (**Fig. 1**), the competitive HXK inhibitor mannoheptulose (Mhl) was added at a concentration of 100 mM to germination medium containing 440 mM Suc and 6 mM of the tested hexose (**Fig. 2A**). Mhl had no effect on the slight Gal-mediated inhibition and only little impact on Fru-mediated and the slight 1F-Fru-mediated inhibition of pollen germination. In contrast, addition of Mhl resulted in full reversion of Glc-mediated inhibition of pollen germination. Furthermore, Mhl resulted in partial reversion of the strong inhibition of pollen germination mediated by Man as evidenced by significantly increased pollen germination from 4.6% without Mhl to 29.5% with Mhl (**Fig. 2A**).

In addition, the Glc-analogs 2-deoxy-Glc (2-DOG), L-Glc, 3-*O*-methyl-Glc (3-OMG), and 6-deoxy-Glc (6-DOG) were tested for their impact on pollen germination at a concentration of 6 mM in Suc-containing medium (**Fig. 2B**). While the phosphorylatable HXK substrate 2-DOG significantly inhibited Suc-supported pollen germination to a similar extent as Man, L-Glc, which is not transported into the cell, did not inhibit pollen germination, supporting a potential intracellular sensing mechanism. In contrast to Glc and the two HXK-phosphorylatable Glc analogs 2-DOG and Man, the non-phosphorylatable Glc analogs 3-OMG and 6-DOG had only a slight effect on Suc-supported pollen germination (**Fig. 2B**), supporting an involvement of a HXK-dependent signaling pathway (Godt et al. 1995, Roitsch et al. 1995, Sinha et al. 2002).

The complementary results deriving from the addition of Mhl to medium containing Suc and different hexoses as well as the analyses of Glc analogs with specific functional features added to Suc-containing medium suggested a potential involvement of HXK as one mediator of the described inhibition of pollen germination. To support these results further, Suc-supported pollen germination and its inhibition by hexoses was

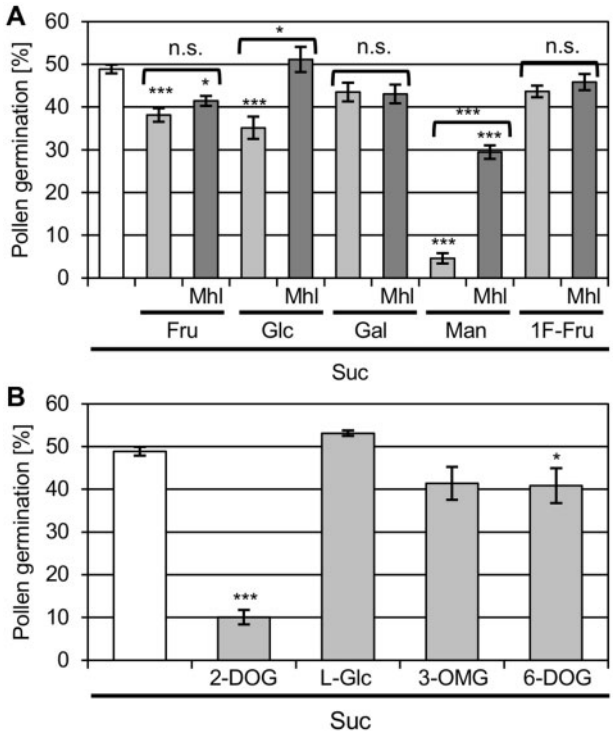


**Fig. 2** Effect of different hexoses, mannoheptulose and glucose analogs on sucrose-mediated pollen germination (PG). (A) Mannoheptulose (Mhl; dark gray columns) neutralizes inhibitory effects of specific hexoses (light gray columns) on Suc-supported PG. (B) Various Glc analogs (gray columns) differentially influence Suc-supported PG. All hexoses and Glc analogs were added at a concentration of 6–440 mM Suc-containing standard medium, Mhl at a concentration of 100 mM. Values (% PG) represent means (± SEM); * and *** indicate significantly different PG at the 0.05 and 0.001 levels of confidence, respectively. Above individual columns, significant differences compared with standard Suc-containing medium (white column) are indicated; above brackets differences between hexose and hexose + Mhl treatment are indicated; n.s., not significant. Fru, fructose; Gal, galactose; Glc, glucose; Man, mannose; 1F-Fru, 1-fluoro-1-deoxy-D-fructose; 2-DOG, 2-deoxyglucose; 3-OMG, 3-*O*-methylglucose; 6-DOG, 6-deoxy-glucose.

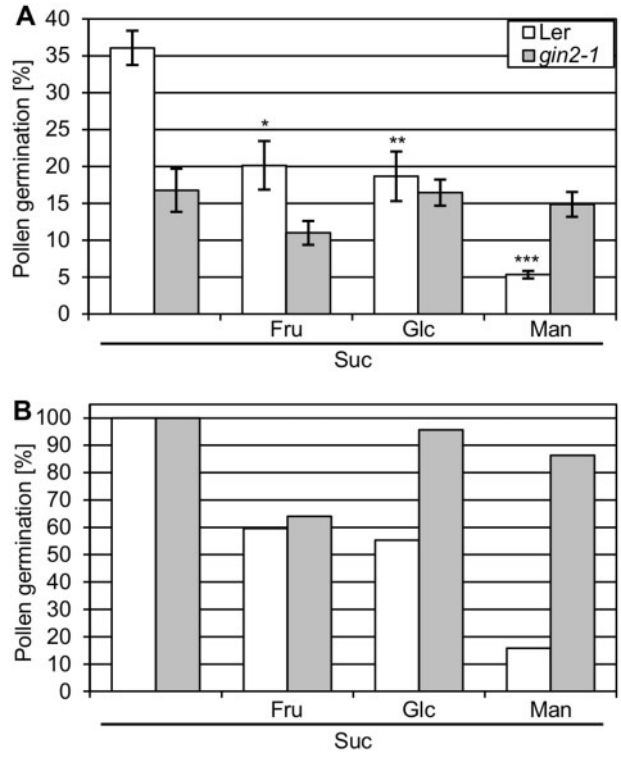


**Fig. 3** Effect of different hexoses on Suc-supported pollen germination (PG) of wild-type (*Ler*; white columns) and the Glc insensitive HXK-deficient mutant *gin2-1* (gray columns). (A) Values (% PG) represent means (± SEM); *, ** and *** indicate significantly different PG compared with standard Suc-containing medium at the 0.05, 0.01 and 0.001 levels of confidence, respectively. (B) Normalized values with PG on Suc-containing medium set to 100% as reference. Hexoses were added at a concentration of 6 mM to 440 mM Suc-containing medium. Fru, fructose; Glc, glucose; Man, mannose; Suc, sucrose.

tested in the HXK1 mutant *gin2-1* (Moore et al. 2003) in comparison with the corresponding *A. thaliana* wild-type Landsberg *erecta* (*Ler*) (**Fig. 3**). Addition of 6 mM of each of Fru, Glc and Man to Suc-containing medium resulted in inhibition of pollen germination in both plant lines. However, the inhibition was in all cases more prominent in the wild type (**Fig. 3A**), as evidenced by significant inhibition of pollen germination in the presence of the different hexoses. In contrast, the hexoses had virtually no impact on Suc-supported pollen germination in *gin2-1*. As the germination rate of *gin2-1* pollen was generally lower compared with *Ler*, pollen germination on the corresponding Suc-containing medium was assigned a value of 100% to allow a direct assessment of the inhibitory effects (**Fig. 3B**). With the exception of Fru, which resulted in similar inhibition of pollen germination in *Ler* and *gin2-1*, the inhibitory effect of Glc and Man on pollen germination was significantly reduced in the Glc-insensitive mutant *gin2-1*. Probably the observed inhibition by Fru is related to partial blockage of some of the sucrose transporters in the fructofuranose form, which is in agreement with the observed inhibitory effect of the sucrose isomers turanose and palatinose (see above). Compared with the wild type, Glc and Man exhibited only approximately 10 and 16% of their inhibitory effect in *gin2-1*, respectively, as evidenced by a pollen germination reduction of 44.7% in *Ler* vs. 4.3% in *gin2-1* for Glc and 84.2% in *Ler* vs. 13.6% in *gin2-1* for Man. The involvement of HXK function in the hexose-dependent inhibition of Suc-supported pollen germination is further evidenced by the effects of the Glc analogs 2-DOG, 3-OMG and 6-DOG in the wild type and *gin2-1* (Supplementary Fig. S3). While the non-phosphorylatable analogs 3-OMG and 6-DOG only show a limited effect on Suc-supported pollen germination in *Ler* and *gin2-1* (comparable with their impact on Col-0 pollen germination), the phosphorylatable 2-DOG strongly inhibits Suc-supported pollen germination in *Ler*. In contrast, this inhibitory effect is minimized and not significantly present in the HXK-deficient mutant *gin2-1*.

Comparably with Suc, cellobiose, maltose, the trisaccharides raffinose, melezitose and the tetrasaccharide stachyose supported pollen germination when provided as exclusive carbohydrate source (**Table 1**). The finding that maltose and cellobiose, but not trehalose, can replace sucrose in the

pollen germination media can be explained by the fact that maltose and cellobiose contain a glucose–glucose (1→4) and trehalose contains a glucose–glucose (1→1) glycosidic linkage and the assumption of a 1→4-specific hydrolase in combination with the 1→2-specific β-fructosidase activity of one of the invertase isoenzymes that accept sucrose and also raffinose, stachyose and melezitose as substrate (Hasegawa and Smolensky 1970, Goetz and Roitsch 1999). The inhibitory potential of 6 and 60 mM Fru, Glc, Gal and Man and 6 mM of the Glc analog 2-DOG were tested in the presence of 440 mM of these sugars in comparison with Suc-supported germination (**Fig. 4**). The inhibitory effect of Fru, Glc, Gal and Man at a concentration of 60 and 6 mM and for 6 mM 2-DOG was evident when added to 440 mM Suc (**Figs. 1, 2A**). However, pollen germination was not significantly inhibited by 6 and 60 mM Fru or Glc when added to medium containing 440 mM cellobiose, maltose or the oligosaccharides (**Fig. 4**) with the exception of a slight inhibitory effect of 6 mM Glc on stachyose-supported pollen germination; in a few cases, Fru and Glc even caused a promotion of pollen germination (**Fig. 4**). This dissimilarity suggests that the sucrose transporters preferentially involved in the internalization of sucrose are different from those exhibiting the highest efficiency for the rest of pollen germination-supportive oligosaccharides. In contrast, 60 mM Gal showed a significant inhibitory effect on pollen germination supported by any of the tested di- and oligosaccharides similar to the effect on Suc-supported pollen germination, while 6 mM Gal only inhibited stachyose-supported pollen germination significantly (**Fig. 4**). Man (60 and 6 mM) and 2-DOG (6 mM) resulted in a significant inhibition of pollen germination in combination with all di- and oligosaccharides tested comparable with their impact on Suc-supported pollen germination (**Fig. 4**). In contrast, 6 mM of the non-phosphorylatable Glc analogs 3-OMG and 6-DOG in general did not influence pollen germination that dramatically: while maltose- and raffinose-supported pollen germination were not inhibited at all and Suc-, cellobiose- and melezitose-supported pollen germination were inhibited by 6-DOG to a certain extent, only stachyose-supported pollen germination was strongly inhibited by both Glc analogs, indicating differential regulation of pollen germination support by the various sugars (Supplementary Fig. S4).

## Discussion

The present study demonstrates that *A. thaliana* pollen germination is differentially regulated by exogenously applied sugars and shows distinct differences between hexoses and certain di- and oligosaccharides.

### Some di- and oligosaccharides support pollen germination

It is well known that pollen germination of *A. thaliana* is supported by Suc (Hülskamp et al. 1995, Stadler et al. 1999) and all experimental protocols for in vitro pollen germination contain Suc as carbon source (e.g. Boavida and McCormick 2007). Our results also show that the disaccharides maltose and cellobiose and the oligosaccharides melezitose, stachyose and raffinose strongly support *A. thaliana* pollen germination. In contrast, the other tested disaccharides either resulted in only poor pollen germination (lactose, leucrose, melibiose and trehalose) or did not support pollen germination at all when added as the sole carbon source to the medium. These results suggest that the nature of the monosaccharide constituents and the type of linkage between them have an influence on the potential of these disaccharides to support pollen germination. Thus, the (1→4)-linked gluco-disaccharides maltose and cellobiose strongly support pollen germination, while replacing the non-reducing glucose moiety by galactose, such as in lactose, drastically decreases the supporting effect on pollen germination. In addition to sucrose, all tested oligosaccharides containing the sucrose motif support pollen germination, while the non-metabolizable dichlorosucrose does not support pollen germination, nor do the sucrose isomers turanose or palatinose. Unlike glucose and sucrose, the non-metabolizable sucrose isomers turanose or palatinose were shown also not to affect photosynthetic activity and Chl fluorescence (Sinha and Roitsch 2002) and activate different signal transduction pathways in tomato (Sinha et al. 2002). These results underline the necessity of accessible carbon to support the highly energy-consuming process of pollen germination.

In *A. thaliana*, sucrose is transported by members of sucrose transporter families that function as sucrose/proton symporters (SUT family; Sauer 2007) and the only recently identified, structurally different class of bidirectional SWEET (sugars will eventually be exported transporter) uniporters (Eom et al. 2015). The SUTs, however, do not exclusively transport Suc but also accept other α-glucosides (maltose) as well as β-glucosides (Sauer 2007). Similarly, *Lilium* pollen germinated well in the presence of sucrose, maltose and cellobiose (Rosen 1968). Thus, the specificity of these transporters can (at least partially) explain the differential impact of the tested di- and oligosaccharides on pollen germination depending on their uptake efficiency via the transporters.

In contrast to the indicated di- and oligosaccharides, hexoses (Fru, Gal, Man and Sor) or monosaccharide alditols (mannitol and sorbitol) did not support pollen germination; only Glc supported pollen germination to a limited extent. Monosaccharides are transported by members of the sugar transport protein (STP) family (Nørholm et al. 2006, Rottmann et al. 2016) and these transporters are important during microspore development and pollen tube growth but not in the first phase of pollen germination (Truernit et al. 1999, Scholz-Starke et al. 2003, Büttner 2007). AtSTP9, a Glc-specific monosaccharide transporter, is very prominently expressed in growing pollen tubes, but only weakly expressed in mature pollen (Schneidereit et al. 2003, Schneidereit et al. 2005). This weak expression could explain the minimal support of pollen germination on Glc, which is in agreement with previous studies in *A. thaliana* (Stadler et al. 1999, Sivitz et al. 2008). Furthermore, AtSTP10 as a high-affinity monosaccharide transporter was shown to be expressed in growing pollen tubes, but not in non-germinated pollen (anthers), and to be especially

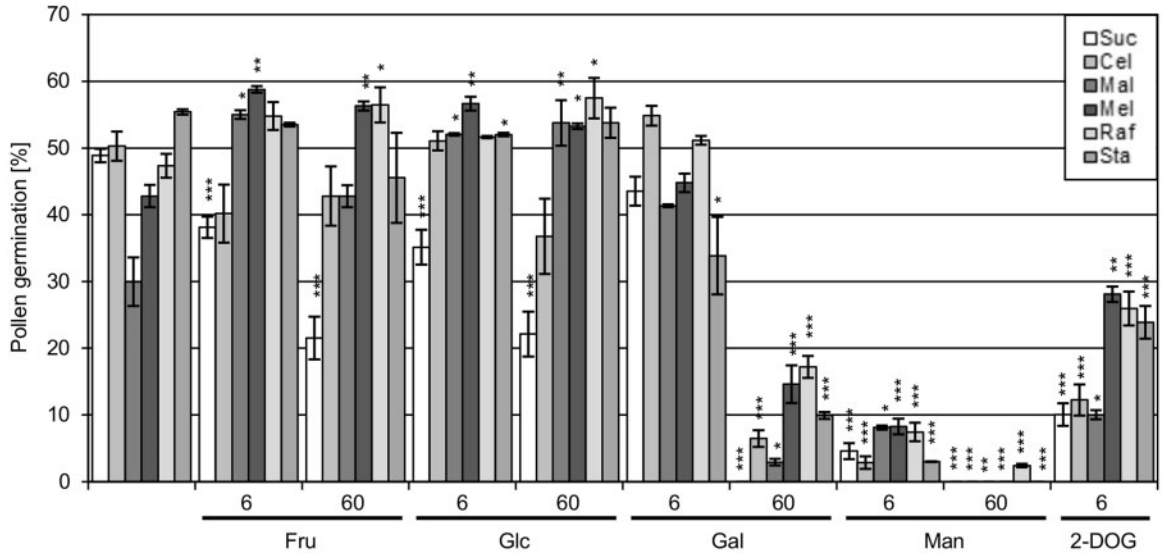


**Fig. 4** Comparison of the inhibitory potential of various monosaccharides on pollen germination (PG) supported by different di- and oligosaccharides. A concentration of 6 or 60 mM of the hexoses fructose (Fru), galactose (Gal), glucose (Glc) and mannose (Man), and 6 mM of the Glc analog 2-deoxyglucose (2-DOG) were added to PG media containing 440 mM of various di- and oligosaccharides. Values (% PG) represent means (± SEM); *, ** and *** indicate significantly different PG compared with the respective standard medium containing only the di- or oligosaccharide at the 0.05, 0.01 and 0.001 levels of confidence, respectively. Cel, cellobiose; Mal, maltose; Mel, melezitose; Raf, raffinose; Sta, stachyose; Suc, sucrose.

regulated by the presence of Glc probably via HXK1-dependent signaling (Reinders 2016, Rottmann et al. 2016). Similarly, in vitro pollen germination of other species such as date palm was more strongly supported by disaccharides as compared with hexoses (Ismail 2014). However, petunia pollen has been shown to germinate well on Glc-containing medium and, when sucrose was supplied, it was hydrolyzed into the hexoses which were taken up by specific transporters (Ylstra et al. 1998) similar to the effect during pearl millet pollen tube growth (Reger et al. 1992). In contrast, *A. thaliana* pollen did not germinate on medium in which Suc was substituted by equimolar concentrations of Fru and Glc (as after Suc hydrolysis; Stadler et al. 1999). Thus, it has to be assumed that the mechanisms involved in the regulation of pollen germination (i.e. uptake of specific sugars) by exogenous carbohydrates differ between plant species.

## Hexokinase is (potentially) involved in glucose- and mannose-mediated inhibition of sucrose-supported pollen germination

The hexoses Fru, Glc, Gal (to a lesser extent) and especially Man caused a reduction of the pollen germination rate even when added at low concentrations of 6 mM to Suc-containing medium (440 mM). This demonstrates that hexoses exert a strong and specific inhibitory effect on pollen germination differing from a potential competitive effect (**Fig. 5**). In contrast, none of the tested disaccharides affected the pollen germination rate comparably with the hexose effect at a concentration of 6 mM in the Suc-containing medium, supporting the specificity of the observed hexose effect.

The involvement of HXK as one pathway in hexose-mediated inhibition of pollen germination (**Fig. 5**) was confirmed by complementary approaches: (i) analyzing the effect of the specific HXK inhibitor Mhl on hexose-mediated pollen germination inhibition; (ii) comparing inhibitory effects of different hexoses and Glc analogs; and (iii) testing hexose-mediated pollen germination inhibition in the HXK-deficient mutant *gin2-1*. Inhibition of HXK by Mhl fully rescued pollen germination after Glc-mediated inhibition, and the reduction of pollen germination caused by Man was also partially neutralized by Mhl. The involvement of HXK was further substantiated by experiments using Glc analogs: only the phosphorylatable HXK substrate analog 2-DOG strongly inhibited Suc-supported pollen germination, while the non-phosphorylatable analogs 3-OMG and 6-DOG had no effect. Also L-Glc, which is not transported into the cell, did not inhibit pollen germination, supporting an intracellular sensing mechanism. Finally, analyses of the HXK mutant *gin2-1* (Moore et al. 2003) also suggested the involvement of HXK in the hexose-mediated inhibition of pollen germination. In *A. thaliana gin2-1*, no significant inhibitory effect of Fru, Glc and Man on Suc-supported pollen germination was found. It is known that HXK plays a central role in sugar metabolism and sugar signaling (Rolland and Sheen 2005, Rolland et al. 2006, Granot et al. 2013, Sheen 2014). Xu et al. (2008) studied the role of OsHXK10 that is exclusively expressed in stamen of rice and demonstrated its essential role in pollen germination. Our results support the important role of HXK as a key enzyme in carbohydrate metabolism, particularly also for the regulation of pollen germination.

The strong effect of Man was striking and seemingly connected to HXK. Inhibitory effects of exogenous Man are well known (Herold and Lewis 1977). Man is phosphorylated by HXK to Man-6-phosphate. However, in *A. thaliana*, there is

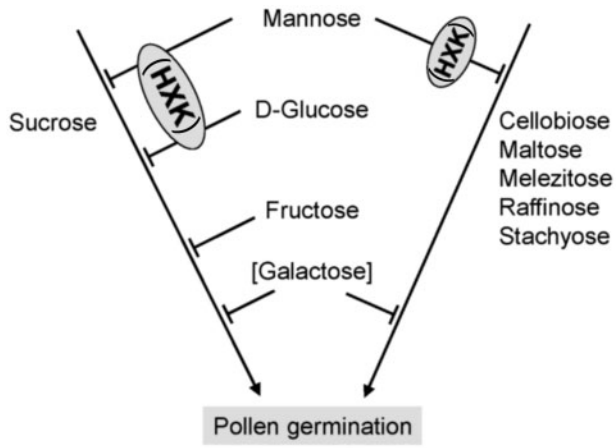


**Fig. 5** Model for the differential regulation of pollen germination by exogenous carbohydrates. Inhibition of sucrose-supported pollen germination (PG) by mannose and glucose potentially involves HXK-dependent signaling, while fructose acts mainly independently of HXK. PG support by other di- as well as oligosaccharides is similarly inhibited by mannose, possibly involving HXK, whereas glucose and fructose seem to inhibit sucrose-supported PG specifically, but not the PG supported by other di- and oligosaccharides. Galactose (at the high concentration of 60 mM) inhibits PG on media containing any of the di- and oligosaccharides, suggesting a different type of inhibition as compared with the other hexoses, potentially involving competition effects. Fructose and potentially galactose sensing requires other signaling pathways.

no further utilization due to a deficiency of the necessary isomerase to convert it to Fru-6-phosphate, and the accumulation of Man-6-phosphate blocks glycolysis and also interferes with phosphate availability (Stein and Hansen 1999). Pego et al. (1999) reported that Man inhibits *A. thaliana* seed germination. They postulated that Man leads to a metabolic signal that is capable of halting germination and that this signal is transmitted via a HXK-mediated pathway and hypothesized that Man is potentially halting the mobilization of seed reserves. HXK is also believed to be involved in pollen germination in the presence of non-Suc di- and oligosaccharides (**Fig. 5**). The strong inhibitory effect of Man on pollen germination on non-Suc media strongly supports this suggestion. The Man-mediated inhibition of pollen germination in the presence of cellobiose, maltose and the tested oligosaccharides was also partially restored by the addition of Mhl (Supplementary Fig. S5).

Different responses to 2-DOG, as compared with other sugars, were also observed by Kojima et al. (2007) for sugar-dependent regulation of ribosomal synthesis and the induction of invertases (Roitsch et al. 1995, Sinha et al. 2002) and sucrose synthase (Godt et al. 1995). The inhibition of pollen germination by 2-DOG could at least partly be attributed to cytotoxic effects. 2-DOG-6-phosphate blocks glycolysis by inhibition of phosphoglucoisomerase, it inhibits protein synthesis and leads to a change in protein glycosylation (Kang and Hwang 2006). In addition, accumulation of 2-DOG-phosphate causes a decrease in ATP content of the cell (Kunze et al. 2001) and, additionally, Dzyubinskaya et al. (2006) observed an activation of apoptosis in guard cells of *A. thaliana* after incubation in 10 mM 2-DOG.

It was striking that the addition of Glc and Fru (6 and 60 mM) caused an inhibition of Suc-supported pollen germination, while they did not alter pollen germination when combined with other pollen germination-promoting di- and oligosaccharides. While HXK phosphorylates Glc and Fru—and to a much lower extent also 1F-Fru (Haradahira et al. 1995)—fructokinase (FRK) phosphorylates only Fru (Granot 2007). Since the affinity of HXK for Fru is orders of magnitude lower than for Glc or Man (Claeyssen and Rivoal 2007, Granot 2007), FRK is most probably responsible for the main part of metabolism of Fru, and Fru signaling is indicated to be mediated by FRUCTOSE INSENSITIVE1/FRUCTOSE-1,6-BISPHOSPHATASE (FINS1/FBP) (Cho and Yoo 2011). FRK activity rises at the end of pollen development (Karni and Aloni 2002); an anther- and pollen-specific expression of FRK was found in tobacco (German et al. 2002), and proteome analysis of rice anthers shows multiple charge isoforms of FRK (Kerim et al. 2003). This is supported by our data as Mhl only slightly neutralized Fru- and 1F-Fru-mediated inhibition of pollen germination and *gin2-1* showed strong inhibition of pollen germination on Fru-containing medium. These results indicate that Fru and 1F-Fru are only partially sensed via HXK, and HXK-independent signaling potentially contributes to the Fru-mediated inhibition of pollen germination, probably involving FINS1/FBP for which a cross-talk with HXK1-mediated Glc signaling has been suggested (Cho and Yoo 2011). Toxic effects, as already discussed for 2-DOG, are thought to be responsible for the complete inhibition of pollen germination at 60 mM. In contrast, the inhibitory effect of Gal on pollen germination is not mediated by HXK, since Gal is known to not be a substrate for HXK (Gonzali et al. 2002), which is supported by the finding that Mhl did not prevent the inhibition at all. Galactokinases, which have already been identified in *Vicia* and *A. thaliana* (Dey 1983, Kaplan et al. 1997), are thought to be involved in this process (Sherson et al. 2003, Blöchl et al. 2007).

In summary, structure-specific differences in their effect on *A. thaliana* pollen germination has been shown for different hexoses, di- and oligosaccharides. While exogenous hexoses seem not to support *A. thaliana* pollen germination, specific di- and oligosaccharides allowed the pollen to germinate, provided that they can be taken up and metabolized. Furthermore, several hexoses were shown to inhibit Suc-supported *A. thaliana* pollen germination, partially involving hexose-specific HXK signaling. The complementary use of sugars exhibiting different structural features, the HXK mutant *gin2-1* and the HXK inhibitor Mhl revealed a sugar-dependent multilayer regulation of *A. thaliana* pollen germination. This possibly comprises specific uptake (e.g. disaccharides over hexoses) as well as metabolic utilization of distinct carbohydrate structures and potentially involves sugar sensing, partially mediated by HXK. Thus, *A. thaliana* pollen germination seems to be a valuable experimental system for future studies addressing sugar sensing and signaling.

## Materials and Methods

### Plant material and cultivation

Pollen from wild-type *A. thaliana* Columbia (Col-0) were used as standard for pollen germination experiments. Additionally, pollen from the mutant line *gin2-1* (Moore et al. 2003) with the corresponding wild-type L*er* were used for some of the experiments. Plants were grown in 'Naturahum' potting soil (Ostendorf Gärtnereierden GmbH) with a 9/15 h 22/18°C day/night cycle (light intensity: 180 µmol $m^{-2}$ $s^{-1}$) until flowering. Soil was treated with Agritox® (Kwizda Agro GmbH) prior to sowing, according to the supplier's instructions.

### Pollen germination

The pollen germination experiments were performed according to Hülskamp et al. (1995), containing 0.4 mM $CaCl_2$ and 0.4 mM $H_3BO_3$ in the final medium. Low melting agarose (1%) was added to 2 × germination medium (without sugar) and heated to dissolve the agarose. This basic medium was stored at −20°C. Sugars were added to aliquots of the medium; 440 mM Suc was used as standard carbon source. The medium was melted at 65°C and two droplets of 50 µl each were placed on a microscope slide which was immediately stored in a humid chamber to prevent desiccation. The slides in the humid chambers were incubated overnight at 26°C. For pollen release, whole flowers were carefully dabbed onto the solidified medium. The percentage of pollen germination (% PG) was defined as the number of germinated pollen grains in the total population of pollen grains analyzed. Pollen with emerging tubes of ≥20 µm was regarded as germinated. Each individual pollen germination test integrated three biological replicates by analyzing pooled pollen from three Col-0 or two L*er* (and *gin2-1*) flowers, respectively. A minimum of 600 pollen grains per single pollen germination experiment were analyzed using a light microscope; for most treatments, at least three independent pollen germination experiments were performed on different days, with exceptions for sugars for which very limited amounts were obtained and particularly in cases when medium containing specific sugar(s) did not support pollen germination at all in two independent experiments. In total, about 300,000 pollen grains were examined in approximatley 500 individual pollen germination experiments testing the effect of 32 different sugars in about 150 different combinations (single or combined supply).

### Chemical synthesis of substituted sugars

In the experiments performed for this study, 32 different sugars were used (Supplementary Fig. S1). The commercially available sugars (2-deoxyglucose, 3-*O*-methylglucose, 6-deoxyglucose, cellobiose, fructose, galactose, D-glucose, L-glucose, mannitol, mannose, sorbitol, L-sorbose, lactose, lactulose, leucrose, maltitol, maltose, mannoheptulose, melezitose, melibiose, raffinose, stachyose, sucralose, sucrose, trehalose and turanose) were purchased from AppliChem, Merck, Carl Roth and Sigma-Aldrich. Glucopyranosyl-mannitol, glucopyranosyl-sorbitol and palatinose were provided from Südzucker.

The non-commercially available sugars were synthesized as follows: 1F-Fru was prepared from 2,3:4,5-di-*O*-isopropylidene β-D-fructopyranose in three steps by formation of the corresponding 1-*O*-trifluoromethanesulfonyl derivative, nucleophilic displacement with tetrabutylammonium fluoride and final aqueous trifluoroacetic acid-mediated hydrolysis of the isopropylidene groups (Haradahira et al. 1995). 1F-Fru thus obtained was shown to exist as a mixture of three tautomeric forms of β-pyranose (71%), β-furanose (21%) and α-furanose (8%) in water (Funcke and von Sonntag 1979). α-D-Fructofuranose-β-D-fructofuranose 1,2′:2,3′-dianhydride (DAF III) was obtained from the pyrolysis of inulin (Blize et al. 1994). 6,6′-Dichloro-6,6′-dideoxysucrose (dichlorosucrose) was prepared from commercial sucrose by reaction with triphenylphosphine and carbon tetrachloride in pyridine (Kashem et al. 1978).

### Statistical analysis

Statistical analyses were performed based on unpaired Student's *t*-test. *P* values ≤ 0.05 were considered significant; in the figures and table *, **, and *** indicate significant differences at the 0.05, 0.01 and 0.001 levels of confidence, respectively, while no label on single columns and n.s. in specific cases indicate no significance.

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## Disclosures

The authors have no conflicts of interest to declare.

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