

# Disaccharide-Mediated Regulation of Sucrose:Fructan-6-Fructosyltransferase, a Key Enzyme of Fructan Synthesis in Barley Leaves<sup>1</sup>

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Previous work has indicated that sugar sensing may be important in the regulation of fructan biosynthesis in grasses. We used primary leaves of barley (*Hordeum vulgare* cv Baraka) to study the mechanisms involved. Excised leaf blades were supplied in the dark with various carbohydrates. Fructan pool sizes and two key enzymes of fructan biosynthesis, sucrose (Suc):Suc-1-fructosyltransferase (1-SST; EC 2.4.1.99) and Suc:fructan-6-fructosyltransferase (6-SFT; EC 2.4.1.10) were analyzed. Upon supply of Suc, fructan pool sizes increased markedly. Within 24 h, 1-SST activity was stimulated by a factor of three and 6-SFT-activity by a factor of more than 20, compared with control leaves supplemented with mannitol (Mit). At the same time, the level of mRNA encoding 6-SFT increased conspicuously. These effects were increased in the presence of the invertase inhibitor 2,5-dideoxy-2,5-imino-D-mannitol. Compared with equimolar solutions of Suc, glucose (Glu) and fructose stimulated 6-SFT activity to a lesser extent. Remarkably, trehalose (Tre; Glc- $\alpha$ -1 and 1- $\alpha$ -Glc) had stimulatory effects on 6-SFT activity and, to a somewhat lesser extent, on 6-SFT mRNA, even in the presence of validoxylamine A, a potent trehalase inhibitor. Tre by itself, however, in the presence or absence of validoxylamine A, did not stimulate fructan accumulation. Monosaccharides phosphorylated by hexokinase but not or weakly metabolized, such as mannose (Man) or 2-deoxy-Glc, had no stimulatory effects on fructan synthesis. When fructose or Man were supplied together with Tre, fructan and starch biosynthesis were strongly stimulated. Concomitantly, phospho-Man isomerase (EC 5.3.1.8) activity was detected. These results indicate that the regulation of fructan synthesis in barley leaves occurs independently of hexokinase and is probably based on the sensing of Suc, and also that the structurally related disaccharide Tre can replace Suc as a regulatory compound.

Fructans (polyfructosyl-Sucs) are the main polysaccharide reserves in vegetative tissues of many grasses, including major crop plants such as wheat and barley (*Hordeum vulgare*) (for reviews, see Pollock and Cairns, 1991; Wiemken et al., 1995; Avigad and Dey, 1997; Vijn and Smeekens, 1999). The first step of their biosynthesis is the transfer of the fructosyl moiety from Suc to a second Suc molecule catalyzed by the enzyme Suc:Suc-1-fructosyltransferase (1-SST; EC 2.4.1.99). The product formed, the trisaccharide 1-kestose, then serves as an acceptor for other fructosyl moieties from Suc and, in all likelihood, these transfer reactions are catalyzed mainly by Suc:fructan-6-fructosyltransferase (6-SFT; EC 2.4.1.10). This enzyme has been cloned and transiently expressed in *Nicotiana plumbaginifolia* protoplasts (Sprenger et al., 1995). It is still controversial whether the very large fructan molecules occurring in certain grasses are exclusively synthesized through this pathway, or if enzymes of a completely different mode of action are involved (see Cairns et al., 1999).

Concerning the regulation of fructan synthesis, it is well known that excised barley leaves accumulate high amounts of fructans when they are exposed to light or incubated in Suc solutions. This is not merely due to a greater abundance of substrate. Previous results indicate that the induction of sucrosyltransferases is crucial for fructan biosynthesis (Wagner et al., 1986; Simmen et al., 1993; Sprenger et al., 1995), but it is unknown so far what control mechanisms are operating. Three mechanisms have been proposed for carbohydrate-mediated gene expression (for review, see Stitt et al., 1995; Koch, 1996). The first, the acetate hypothesis, is based on observations that hexoses degradable by glycolysis (Glu and Fru) and acetate repress gluconeogenic enzymes in cucumber cell cultures. Hexoses that are substrates for hexokinase but are not or only slowly degraded (mannose [Man] and 2-deoxy-Glc [DOG]) and dicarboxylic acids (malate and succinate), on the other hand, do not repress these enzymes (Graham et al., 1994).

The second theory is that sugar-mediated changes in gene expression may depend on phosphorylation of Glu by hexokinase (Graham et al., 1994; Jang and Sheen, 1994). Plants that express an Arabidopsis hexokinase (*AtHXK*) antisense construct, and thus have reduced levels of hexokinase, are less sensitive to Glu than wild-type plants. While plants overexpressing hexokinase are hypersensitive to Glu, they show se-

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verely stunted hypocotyl growth and do not green (Jang et al., 1997). The plants overexpressing hexokinase are also sensitive to Glc derivatives that are phosphorylated by hexokinase, e.g. DOG, but not to the ones that cannot be phosphorylated by hexokinase, e.g. 3-O-methyl-Glc (Smeekens, 1998).

The third theory is that Suc or similar disaccharides are involved in carbohydrate-mediated expression of certain genes before the disaccharides are cleaved and further metabolized (see Koch, 1996). Thus, in some cases, Suc has been shown to affect expression of certain genes (e.g. Wenzler et al., 1989; Jefferson et al., 1990; Koch et al., 1992; Ishiguro and Nakamura, 1994; Chiou and Bush, 1998; Rook et al., 1998). Clearly, the major problem of experiments applying Suc is the fact that Suc may be efficiently cleaved by invertases. Thus, it is difficult to distinguish between effects due to Suc itself or to Glu or Fru. Potent invertase inhibitors are known but not commercially available (but see Legler et al., 1993). A way to avoid this problem could be to feed Suc analogs that are not cleaved by invertases, such as fluoro-Suc (Hitz et al., 1985). One possible analog is also trehalose (Tre;  $\alpha$ -D-glucopyranosyl-[1,1]- $\alpha$ -D-glucopyranoside), a disaccharide of widespread occurrence in many microorganisms, where it plays a role in storage of reduced carbon and also acts as an important protectant against the deleterious effects of various stresses (Wiemken, 1990; Crowe et al., 1998). In general, Tre is believed not to be synthesized by higher plants (see Müller et al., 1995a, 1999b). However, although Tre is not cleaved by plant invertases, it is degraded by trehalases, which are very common in plants and are probably located in the apoplast (see Müller et al., 1995a; Aeschbacher et al., 1999). These trehalases can be very efficiently inhibited *in vivo* by validamycin A, a powerful and very specific trehalase inhibitor (see Müller et al., 1995b, and refs. therein, 1998; Goddijn et al., 1997).

Recently, we have shown that in sterile soybean roots, Suc synthase is stimulated not only upon feeding Suc, but also upon feeding Tre, even in the presence of validamycin A, whereas feeding Glu has no marked effect (Müller et al., 1998). These results indicate that Tre could indeed be a useful tool to study carbohydrate-mediated gene expression. Previously, a stimulation of global Suc:Suc fructosyltransferase activity by Tre was found in excised barley leaves incubated in the dark (Wagner and Wiemken, 1986; Wagner et al., 1986). Tre stood out among the carbohydrates tested, since it was the only one stimulating fructosyltransferase activity without simultaneously causing the accumulation of fructans. We present new results showing that among the fructosyltransferases, 6-SFT, the supposed key enzyme for mass production of fructan synthesis in barley leaves (see Wiemken et al., 1995), is particularly strongly induced by the external application of Suc or by Tre. To prevent a possible degradation of Tre by endogenous

trehalases (EC 3.2.1.28), we have included the potent trehalase inhibitor validoxylamine A (Vox), the deglycosylated form of validamycin A, which has been shown to be very efficient in trehalase inhibition studies *in vivo* (Kono et al., 1993).

## RESULTS

### Fructans and Suc:Fructosyltransferases in Excised Barley Leaves

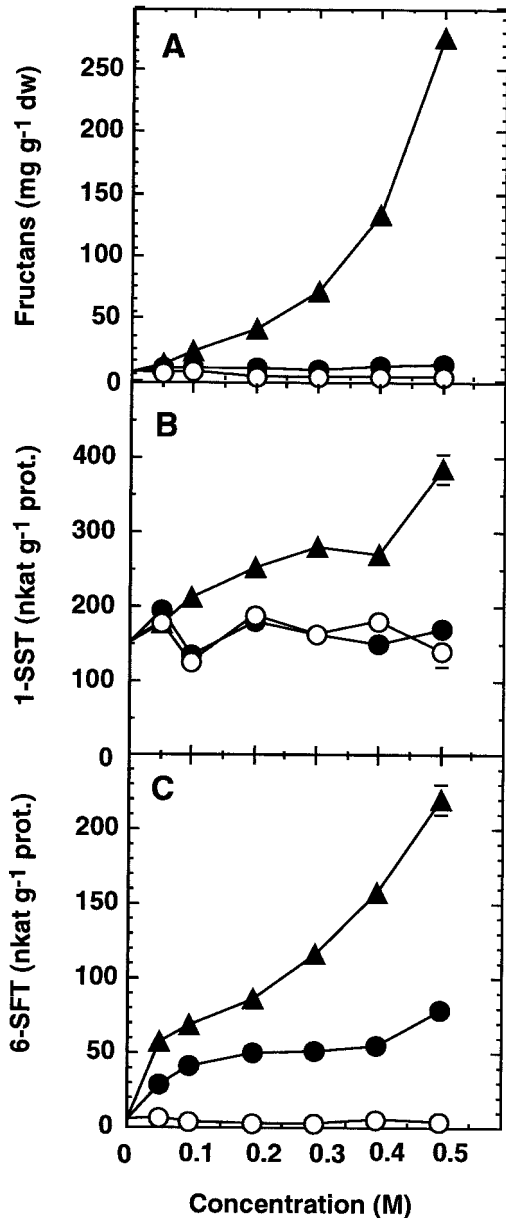
Leaf blades from 14-d-old primary leaves contained only small amounts of fructans (less than 0.4% dry weight; Table I). In order to compare the induction of fructan biosynthesis by Suc with the induction by Tre, leaf blades were excised and dipped with their proximal end in up to 0.5 M Suc or Tre solutions and incubated in the dark for 24 h. Mannitol (Mit) was included as a negative control. Since it was already well known that metabolizable monosaccharides (Glc and Fru) that can be transformed to Suc induce fructan biosynthesis, treatments with these carbohydrates were not included at this stage. Upon feeding of Suc, fructan contents increased in a concentration-dependent manner, reaching 28% of dry weight in a 0.5 M solution (Fig. 1A). In leaf blades incubated in Mit, fructan pool sizes remained similar to zero-time control leaves. When Tre was supplied, the increase of fructan was negligible.

Furthermore, fructosyl transferase activities were measured from desalted crude extracts. Activity of 1-SST had an initial level of about 0.13  $\mu$ kat  $g^{-1}$  protein in extracts from the zero-time control leaves of these unfertilized seedlings (Table I). This activity increased in Suc-treated leaves to 0.38  $\mu$ kat  $g^{-1}$  protein at 0.5 M Suc. Treatment with Mit and Tre did not affect this activity markedly (Fig. 1B). However, 6-SFT was only at background levels (less than 6 nkat  $g^{-1}$  protein) in zero-time control (Table I) and Mit-treated leaves. Upon feeding Suc, this activity increased in a concentration-dependent manner to reach more than 200 nkat  $g^{-1}$  protein at 0.5 M Suc. Tre also stimulated this activity markedly, but to a lesser extent (Fig. 1C).

**Table I.** Suc:fructosyltransferase and trehalase activities and storage carbohydrates in leaf blades of 14-d-old barley

Enzyme activities and carbohydrate contents were analyzed in lyophilized leaf powder. Mean values and sds are given for five independent samples.

Parameter	Unit	Content
Enzymes		
Invertase	$\mu$ kat $g^{-1}$ protein	6.5 $\pm$ 0.8
1-SST	nkat $g^{-1}$ protein	134.2 $\pm$ 6.1
6-SFT	nkat $g^{-1}$ protein	5.0 $\pm$ 0.2
Trehalase	nkat $g^{-1}$ protein	14.1 $\pm$ 2.1
Storage carbohydrates		
Fructans	mg $g^{-1}$ dry wt	3.6 $\pm$ 0.9
Starch	mg $g^{-1}$ dry wt	4.9 $\pm$ 1.3



**Figure 1.** Fructan contents (A), 1-SST (B), and 6-SFT (C) activities in excised primary leaf blades of 14-d-old barley incubated for 24 h in solutions of different carbohydrates (Mit [○], Tre [●], and Suc [▲]) at different concentrations. Mean values  $\pm$  SE are given for three independent samples. A, Fructans; B, 1-SST; C, 6-SFT.

### Trehalase in Barley Leaves

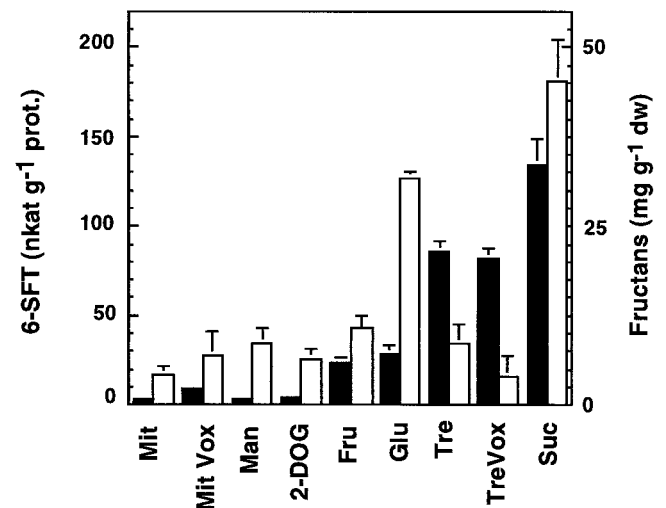
Was the stimulation of 6-SFT by Tre due to Tre itself or to a catabolite (e.g. Glu)? Tre cannot be cleaved by invertases, but by specific trehalase found in many plants (see Müller et al., 1995a). The 14-d-old barley leaves contained a base level of trehalases of approximately  $14 \text{ nkat g}^{-1}$  protein. Interestingly, this activity increased in leaves treated with  $0.5 \text{ M}$  Tre up to  $64 \pm 7.5 \text{ nkat g}^{-1}$  protein, compared with  $33 \pm 1.7 \text{ nkat g}^{-1}$  protein in  $0.5 \text{ M}$  Mit-treated and to  $31 \pm 1 \text{ nkat g}^{-1}$  protein in  $0.5 \text{ M}$  Suc-treated leaves. We

showed previously (Müller et al., 1995b) that by adding a potent trehalase inhibitor, validamycin A, in micromolar concentrations to Tre, Tre degradation could be inhibited efficiently in planta.

Therefore, in a second series of experiments, excised primary barley leaves were supplemented with various carbohydrates ( $0.2 \text{ M}$ ) including Tre with and without Vox, the aglucon of validamycin A. To determine whether the inhibition of trehalase by validamycin A observed in soybean and cowpea (Müller et al., 1995b) could be reproduced in barley with Vox, excised barley leaves were fed with Tre for 24 h; thereafter, Tre contents were  $56 \pm 4.7 \text{ mg g}^{-1}$  dry weight. Upon feeding Vox in addition to Tre, this value was twice as high, namely  $111 \pm 8.9 \text{ mg g}^{-1}$  dry weight. This increase was highly significant ( $P < 0.0001$ ;  $t$  test). In the same leaves, trehalase activity was measured in crude extracts. It decreased from  $55 \pm 4.2 \text{ nkat g}^{-1}$  protein in leaves fed with Tre alone to levels below the detection limit in leaves supplied in addition with Vox. Thus, Vox was a suitable inhibitor for trehalase activity in barley leaves and was included in the further experiments together with Tre.

### 6-SFT Is Stimulated by Suc and Tre

In a series of experiments, monosaccharides (Fru, Glu, DOG, and Man) susceptible to being phosphorylated by hexokinase were compared with Suc and Tre added alone or in combination with Vox with respect to the potential to stimulate fructosyl transferases in the leaves. In leaves incubated with Mit, 6-SFT remained at background levels (approximately  $4 \text{ nkat g}^{-1}$  protein). The same range of values was observed upon feeding Man or DOG (Fig. 2). Fru and Glu significantly stimulated 6-SFT ( $23$  and  $30 \text{ nkat g}^{-1}$  protein)



**Figure 2.** Activity of 6-SFT (black bars) and fructan contents (white bars) in excised primary leaf blades of 14-d-old barley incubated for 24 h in  $0.2 \text{ M}$  solutions of different carbohydrates. Where indicated,  $10 \mu\text{M}$  Vox was added. Mean values  $\pm$  SE are given for at least six independent samples.

**Table II.** Effects of Dim and Vox on the induction of fructan biosynthesis by externally applied Suc

Primary leaf blades of 14-d-old barley were excised and incubated for 24 h in the dark in a 0.2 M solution of Mit or Suc supplemented with Dim or Vox as indicated. Mean values  $\pm$  SE correspond to three independent samples. Different letters indicate significantly different values ( $P < 0.05$ ; ANOVA followed by Student-Newman-Keuls-test).

Treatment	Suc	Fructans	Invertase	1-SST	6-SFT
	<i>mg g<sup>-1</sup> dry wt</i>		<i>nkcat mg<sup>-1</sup> protein</i>		
Mit	3.0 $\pm$ 1.4a	4.1 $\pm$ 0.8a	3.2 $\pm$ 0.3a	92 $\pm$ 18a	5 $\pm$ 0.9a
Mit + Vox (10 $\mu$ M)	1.7 $\pm$ 0.7a	3.3 $\pm$ 0.3a	3.4 $\pm$ 0.8	96 $\pm$ 6a	5 $\pm$ 1.6a
Mit + Dim (2 mM)	2.4 $\pm$ 0.9a	8.1 $\pm$ 1.3b	3.7 $\pm$ 0.6	130 $\pm$ 23ab	5 $\pm$ 0.6a
Suc	17.5 $\pm$ 1.5b	44.3 $\pm$ 5.5c	3.5 $\pm$ 0.4	217 $\pm$ 24bc	106 $\pm$ 6.9b
Suc + Vox (10 $\mu$ M)	18.5 $\pm$ 1.6b	44.8 $\pm$ 8.7c	3.1 $\pm$ 0.7	225 $\pm$ 36bc	111 $\pm$ 7.9b
Suc + Dim (2 mM)	44.9 $\pm$ 2.8c	123.5 $\pm$ 9.3d	4.8 $\pm$ 0.9a	295 $\pm$ 53c	208 $\pm$ 21.1c

$g^{-1}$  protein in mean, respectively). The highest stimulations were observed upon feeding Tre (approximately 85 nkat  $g^{-1}$  protein) and Suc (134 nkat  $g^{-1}$  protein). The addition of Vox did not change the stimulatory effect of Tre. The effects of Tre (with or without Vox) were much higher than those of Glu or Fru (ANOVA,  $P < 0.01$ ; Fig. 2). As expected, fructan contents were not increased above the background level by Tre. When Vox was supplied in addition to Tre, fructan contents were even lower. Only on Glu and Suc, were fructan contents above 2% dry weight observed (Fig. 2). These results were different from previously published ones (Wagner et al., 1986) in which Fru fueled fructan biosynthesis more efficiently than Glu.

In another series of experiments, we investigated whether Suc in the presence of an invertase inhibitor had similar stimulatory effects on fructan biosynthesis. A suitable inhibitor for such studies was Dim, which inhibited yeast invertase in a micromolar range (Legler et al., 1993). Upon feeding 2 mM Dim together with Suc, Suc increased to more than twice as much as upon feeding Suc alone (Table II). Fructans were nearly three times as high in the presence of Dim as without Dim. Furthermore, issuing from desalted crude extracts, invertase and fructosyl transferase activities were investigated. Total invertase activity was between 3 and 5  $\mu$ kat  $g^{-1}$  protein and did not differ significantly between treatments. Concerning transferase activities, 1-SST was significantly stimulated by Suc compared with Mit. Co-supplementation of Dim had no significant additive effects. Conversely, 6-SFT was stimulated nearly twice as much in the presence of Dim compared with Suc alone. The trehalase inhibitor Vox did not alter the effects of Suc (Table II).

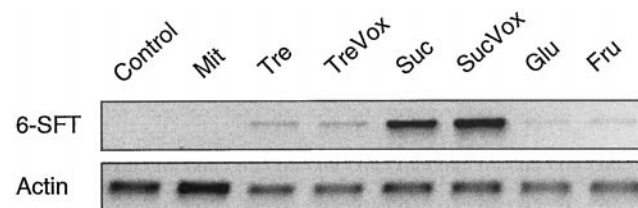
#### Expression of the Gene Encoding 6-SFT

Based on these observations, we wanted to analyze whether Tre could affect the expression of the gene encoding 6-SFT. The genes encoding for barley 6-SFT (Sprenger et al., 1995) had been cloned and sequenced, so suitable probes could be easily obtained. Using a RNA-blot approach (northern blot), no sig-

nals could be detected in a reproducible way. Therefore, a semiquantitative PCR approach was chosen, which included actin as a marker for constitutively induced genes. 6-SFT transcripts were undetectable in cDNA from zero-time control and mannitol-treated leaves. In leaves treated with Glc or Fru, only weak signals were detected, whereas leaves treated with Tre or with Tre together with Vox yielded stronger signals. The highest expression level was observed in leaves fed with Suc. Feeding Suc together with Vox did not affect this pattern (Fig. 3).

#### Tre Is a Trigger for Directing Metabolism to Fructan and Starch Accumulation

In the previously described experiments, the accumulation of fructans was only observed upon feeding of Suc and, to a lesser extent, upon feeding of Glu (Fig. 2). Tre was remarkable among the carbohydrates tested in that it efficiently induced 6-SFT, but was not degraded enough for fueling fructan accumulation, even when no trehalase inhibitor was supplied. This prompted experiments where Tre was supplied together with monosaccharides inducing 6-SFT only weakly, namely Fru and Man (0.2 M each); Suc was included as a positive control. Treatments with Glu were not included, since Glu alone induced fructan biosynthesis quite efficiently in previous experiments (Fig. 2). To avoid degradation of Tre, the trehalase inhibitor Vox (10  $\mu$ M) was also added. When Tre was fed together with Mit, fructans remained at zero-time control levels (approximately 5 mg  $g^{-1}$  dry weight). The same was observed when

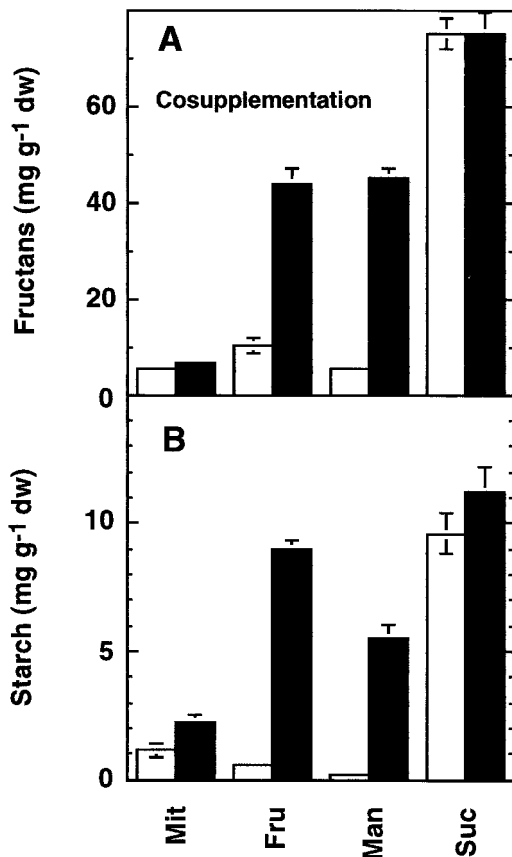


**Figure 3.** Levels of mRNAs of 6-SFT and actin in excised, primary leaf blades of barley incubated in solutions of different carbohydrates, as detected by reverse transcriptase-PCR reactions.

Fru or Man were fed together with Mit. Only Suc supplied with Mit induced fructan accumulation (Fig. 4A).

When Fru and, surprisingly, Man were fed together with Tre, however, fructans were conspicuously increased to approximately  $45 \text{ mg g}^{-1}$  dry weight, thus reaching more than one-half of the amount observed upon feeding of Suc. Suc together with Tre instead of Mit did not increase fructan biosynthesis further, indicating that the amount of Suc was limiting (Fig. 4A compare with Fig. 1). Interestingly, these stimulating effects by Tre were not restricted to the synthesis of fructans. Starch, the second major storage carbohydrate in barley leaf blades, had pool sizes between approximately  $2 \text{ mg g}^{-1}$  dry weight in Mit-treated leaf blades and approximately  $10 \text{ mg g}^{-1}$  dry weight in Suc-treated ones. Feeding of Fru or Man alone was again not sufficient to induce filling of the starch pools. However, upon feeding Fru or Man in addition to Tre, starch contents were significantly increased, approximating values obtained in the Suc-fed leaf blades (Fig. 4B).

The observation that feeding of Fru or Man together with Tre in the presence of a trehalase inhib-



**Figure 4.** Fructan (A) and starch (B) contents in excised primary leaf blades of 14-d-old barley incubated for 24 h in 0.2 M solutions of different carbohydrates, supplemented with either mannitol (Mit, 0.2 M; white columns) or Tre (0.2 M + 10  $\mu\text{M}$  Vox; black columns). Mean values  $\pm$  SE are given for five independent samples.

**Table III.** Phospho-Man and phospho-Glc isomerase activities in barley leaf blades

Primary leaf blades of 14-d-old barley were excised and incubated for 24 h in the dark in a 0.2 M solution of Man supplemented with 0.2 M Mit or Tre together with 10  $\mu\text{M}$  Vox. Isomerase activities were analyzed as described in "Materials and Methods." Mean values  $\pm$  SE correspond to four independent samples. Data were analyzed by ANOVA followed by a Student-Newman-Keuls test.

Treatment	Phospho-Man Isomerase	Phospho-Glc Isomerase
	$\text{nkat g}^{-1} \text{ protein}$	$\mu\text{kat g}^{-1} \text{ protein}$
ManTre	$3.05 \pm 0.56$	$0.5 \pm 0.02$
ManMit	<0.5	$0.4 \pm 0.02$
<i>P</i>	<0.001	<0.05

itor led to an accumulation of fructans and starch was quite surprising. It is very unlikely that Tre provided the carbon skeletons for these compounds, since trehalase was completely inhibited in the presence of Vox (see above). To explain the effects of Man, we wanted to investigate whether barley leaves contained phospho-Man isomerase activity and if this activity was stimulated by Tre. For this purpose, barley leaf blades were supplied with 0.2 M Man and Tre or Mit in the presence of 10  $\mu\text{M}$  Vox. After 24 h, leaf blades were harvested, and phospho-Man- and phospho-Glc-isomerase activities were analyzed (Table III). Interestingly, leaf blades treated with Man and Tre contained low but detectable phospho-Man isomerase activity. In leaf blades treated with Man and Mit, this activity was below the detection limit. As expected, phospho-Glc-isomerase activity was more than 100 times higher than phospho-Man isomerase activity. In the leaf blades treated with Man and Tre, this activity was slightly but significantly ( $P < 0.05$ ) higher than in Man- and Mit-treated leaf blades (Table III).

## DISCUSSION

Our data indicate that soluble carbohydrates are strong regulators of 6-SFT, one of the key enzymes responsible for de novo fructan synthesis in barley leaves. In contrast to previously published studies (Wagner et al., 1986), 1-SST, the enzyme believed to be responsible for triggering fructan synthesis in barley leaves (Wiemken et al., 1995), is already induced at the stage of excision, and thereafter increases only by a factor of three upon feeding of Suc and not at all upon feeding of Tre (compared with a factor of 15 in Wagner et al., 1986). This is in all likelihood due to a slightly different state of leaf development, since it is well known that the basic level and the inducibility of 1-SST activity strongly depends on the developmental stage of the leaf (Wagner and Wiemken 1989). Furthermore, the different growth conditions of the seedlings could be the cause of the differences.

In previous studies, seedlings have been grown on fertile commercial soil mixtures, whereas in this study, vermiculite without any fertilizer was used as

substrate. Under conditions of mineral nutrient deficiency, fructan synthesis is known to be induced in barley leaves (see Wang and Tillberg 1996; J. Müller, R.A. Aeschbacher, N. Sprenger, T. Boller, and A. Wiemken, unpublished results). Thus, leaves used in the present study were already preconditioned for fructan synthesis at the stage of excision. In these leaves, the triggering enzyme, 1-SST, was already induced to a quite high degree and, after excision, a very rapid induction of 6-SFT takes place. As expected, the highest effect on induction of 6-SFT activity and expression levels is observed after feeding leaf blades with Suc, the natural substrate of fructan biosynthesis. However, most interestingly, the non-reducing disaccharide Tre, which is only weakly metabolizable by higher plants (see Müller et al., 1995a, 1995b, 1999b) and therefore does not furnish Suc as a substrate for fructan synthesis, effectively stimulated 6-SFT activity and the expression of its mRNA. This stimulation was not impaired upon supply of Tre in combination with the strong trehalase inhibitor Vox, ruling out the possibility that Tre acts only after degradation to Glu (see also Müller et al., 1995b). Moreover, Glu and Fru, the direct catabolites of Suc and Tre, have much weaker effects on 6-SFT induction than the two disaccharides at equimolar concentrations.

Monosaccharides that can be phosphorylated by hexokinase but are normally not or only slowly catabolized (such as Man and DOG) have no effects. This indicates a sensing of disaccharides independently of hexokinase (Jang et al., 1997). The fact that an invertase inhibitor increases the effects of Suc points in the same direction. Concerning the action of these inhibitors, one has to take into account that extracellular invertases and trehalases are certainly targets reached by these inhibitors, while it is as yet not clear if intracellular enzymes are also inhibited. However, trehalases appear to be apoplastic in many plant organs (J. Müller and L. Schellenbaum, unpublished results; Müller et al., 1995a).

On the basis of these results and those published previously (Wagner et al., 1986; Müller et al., 1998), we suggest that Tre may act as a Suc analog in carbohydrate-mediated gene expression. The marked growth effects observed in transgenic plants expressing microbial Tre biosynthesis genes may be caused by an interference of Tre in sugar sensing (Goddijn et al., 1997; Goddijn and van Dun, 1999). These observations and the results shown here indicate that the presence of Tre in plant tissues due to external supply or to expression of a transgene could trigger fluxes of carbohydrates to alternative pools. The following models can be used to explain the regulatory role of Suc. The first model is that the flux of Suc mediated by a Suc transporter or by catabolic enzymes such as invertases might be sensed (see Lalonde et al., 1999). Tre or other non-metabolized analogs of Suc could inhibit a Suc-metabolizing enzyme or a trans-

porter and, thus, change Suc fluxes between or within compartments. We have observed only small inhibitory effects of Tre on invertase, 1-SST, or 6-SFT activities from barley leaves (J. Müller, R. Aeschbacher, N. Sprenger, T. Boller, and A. Wiemken, unpublished results).

In the second model, Suc, or analogs of it, interact with a receptor located in the apoplast or on the plasma membrane and influence gene expression through second messengers such as calcium (Ohto et al., 1995; Ohto and Nakamura, 1995). Calcium-dependent protein kinases could then activate or inactivate Suc-metabolizing enzymes or transporters directly through phosphorylation; they could interact with transcription factors such as the SP8-binding factor, which has been shown to bind to specific motifs in the promoter regions of some Suc-inducible genes (Ishiguro and Nakamura, 1994; for review, see Smeekens, 1998); or they could interact with the mRNA (Chan and Yu, 1998).

The third model is that Suc or Suc analogs could interact with an intranuclear DNA-binding protein, thus directly triggering transcription of specific genes. This model corresponds to the mode of induction of the lac operon in *Escherichia coli* (Sambrook et al., 1989). To our knowledge, it has not yet been shown whether known plant transcription factors are able to interact directly with carbohydrates. The prerequisite for the validity of this last hypothesis is that Suc and the effective analogs cross the plasma membrane. Indeed, Suc is in general taken up more efficiently than Glu and especially Fru (e.g. Reinhold and Kaplan, 1984). In barley, it has been shown using protoplasts that Tre is taken up in addition to Suc (Wagner and Wiemken, 1986; for further comments, see Müller et al., 1999b).

The biochemical properties of Tre uptake are not yet known. The finding that soybean plants supplied with Tre in their intact root system accumulate Tre in their leaves if validamycin is also supplied indicates, however, that both compounds can be translocated symplastically. Monosaccharides (Glu and Fru) could have an effect independently of hexokinase, namely as substrates for resynthesis of Suc. This has been shown to occur instantaneously at high rates upon feeding of these monosaccharides to barley leaf protoplasts (Wagner and Wiemken, 1986). Together with the lower uptake rate compared with Suc, this could explain why these monosaccharides have weaker effects on fructan biosynthesis than Suc.

A surprising result was that Tre stimulates the metabolism of Man, apparently by inducing phospho-Man isomerase; phospho-Glc isomerase may also be slightly stimulated. This suggests that Tre and perhaps other disaccharides may not only interfere with the regulation of synthesis of carbohydrate reserves such as starch and fructans, but may also act on monosaccharide interconversion and glycolysis. Further research is needed to elucidate if one of these

models is valid or if Suc and related compounds act on gene expression in a more indirect way, such as through an interplay with phytohormones (Zhou et al., 1998).

## MATERIALS AND METHODS

### Plant Material

Seeds of barley (*Hordeum vulgare* L. cv Baraka) were germinated and grown for 2 weeks in sterile vermiculite (Vermica SA, Bözen, Switzerland). The growth conditions were 18-h day at 81  $\mu$ E and 22°C, 6-h night at 18°C, 70% relative humidity. Under these conditions, plants had a fully expanded primary leaf and an emerging secondary leaf. The leaf blades (about 5 cm in length) were excised from the primary leaves and put in 50-mL Erlenmeyer flasks (six blades per flask) containing 15 mL of carbohydrate solutions supplemented or not with 10  $\mu$ L of Vox as indicated. The Erlenmeyer flasks were incubated for 24 h in an desiccator containing solid NaOH (1 g L<sup>-1</sup> desiccator volume) at 20°C in the dark (Wagner et al., 1986). Immediately after harvesting, the leaf blades were frozen in liquid nitrogen and stored at -70°C. For the analysis of carbohydrates and enzyme activities, the leaves were either used directly or lyophilized and ground to a fine powder. Aliquots of this powder were used for the subsequent analyses.

### Enzyme Analysis

Invertase and fructosyltransferase activities from barley were analyzed using desalted crude extracts as described previously (Müller et al., 1999a). Trehalase activity was assayed as described previously (Müller et al., 1995b). In all assays, values were corrected for enzyme and substrate blanks. Soluble protein was analyzed according to the method of Bradford (1976).

In order to assay phospho-Glc isomerase (EC 5.3.1.9) and phospho-Man isomerase (EC 5.3.1.8), frozen leaf blades were extracted and proteins were ammonium sulfate precipitated as described earlier (Müller et al., 1998). Both enzymes were analyzed by a coupled assay in microtiter plates according to a protocol published previously (Bergmeyer, 1974). Phospho-Glc isomerase was assayed in 100 mM morpholinopropane sulfonic acid/K<sup>+</sup>, pH 7.6, containing 1.4 mM Fru-6-P, 10 mM MgCl<sub>2</sub>, 0.4 mM NADP, and 2 nkat of Glc-6-P dehydrogenase (from *Leuconostoc mesenteroides*, Fluka, Buchs, Switzerland) in a total volume of 0.2 mL. Phospho-Man isomerase was assayed in the same buffer as above containing 3.2 mM Man-6-P (Sigma, Buchs, Switzerland), 0.4 mM NADP, 2 nkat of phospho-Glc isomerase (from yeast, Boehringer Mannheim, Germany), and 2 nkat of Glc-6-P dehydrogenase (from *L. mesenteroides*, Fluka) in a total volume of 0.2 mL. All assays were corrected for enzyme and substrate blanks.

### Analysis of Carbohydrates

Total soluble carbohydrates were extracted as described previously (Müller et al., 1999a). Total soluble carbohy-

drates and the products formed by the enzyme reactions described above were analyzed by anion-exchange HPLC as described previously (Müller et al., 1999a). Starch was analyzed by enzymatic hydrolysis and determination of Glu by HPLC as described previously (Müller et al., 1999a).

### Molecular Techniques

If not otherwise mentioned, standard molecular biology techniques were performed according to the method of Ausubel et al. (1992) and Sambrook et al. (1989). For reverse transcriptase-PCR (Bloch, 1991), total RNA was extracted from barley leaves using the hot-phenol method as described (Mohr et al., 1998). Potential DNA contaminations were removed by treating the RNA with DNase I using a kit (MessageClean, GenHunter Corporation, Nashville, TN). One microgram of RNA of this preparation was reverse-transcribed using a kit (Boehringer Mannheim) with both a random and an oligo(dT) primer. One microliter of the cDNA preparations was used per PCR reaction in a total volume of 30  $\mu$ L. Actin was amplified with 27 cycles. Primers used for the amplification were designed to have similar annealing temperatures. For 6-SFT, the primer o13457 5'-GTCCACCTCCTTCTA TGATC-3' and primer o13283 5'-TTTCCGCCGTC ACGCTGGCAC-3' (502 bp; accession no. X83233) was used. For the amplification of actin cDNAs, degenerate primers were synthesized. Primer oActF 5'-GTNYTNGAYWSNNGNGAYGG-3' and oActR 5'-TCNGCDATNCCNGGRA ACAT-3' were used with inosines incorporated at positions where the nucleotide identity is completely ambiguous (positions N). These primers map two regions conserved in a multitude of actin genes from plants of different origin (e.g. accession nos. U60506 and AF002687). Primer oActF maps to the conserved actin protein sequence VLDSGDG, and primer oActR maps to the sequence TMFPGIA in the reverse direction. The predicted size of amplified actin fragments is about 495 bp. Degenerate primers were used, since amplification of several actin cDNAs allows for a more accurate comparison of the success of cDNA synthesis between individual samples than when amplifying a single actin gene alone.

### Chemicals

If not indicated otherwise, all chemicals were purchased from Fluka. Vox was a gift from Novartis (Basel). 2,5-Dideoxy-2,5-imino-D-mannitol (Dim) was a gift from Dr. G. Legler (University of Cologne, Germany).

### Statistics

Analyses of variance and Student-Newman-Keuls tests were performed using the software SigmaStat (Jandel Scientific, San Rafael, CA).

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