# Glucose and Disaccharide-Sensing Mechanisms Modulate the Expression of *α-amylase* in Barley Embryos[1]

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The aim of this study was to investigate the sugar-sensing processes modulating the expression of *α-amylase* in barley (*Hordeum vulgaris* L. var Himalaya) embryos. The results highlight the existence of independent glucose (Glc) and disaccharides sensing. Glc treatment destabilizes the *α-amylase* mRNA. Non-metabolizable disaccharides repress *α-amylase* induction, but have no effects on transcript stability. Structure-function analysis indicates that a fructose (Fru) moiety is needed for disaccharide sensing. Lactulose (β-galactose [Gal][1→4]Fru), palatinose (Glc[1→6]Fru), and turanose (Glc[1→3]Fru) are not metabolized but repress *α-amylase*. Disrupting the fructosyl moiety of lactulose and palatinose, or replacing the Fru moiety of β-Gal[1→4]Fru with Glc or Gal results in molecules unable to repress *α-amylase*. Comparison of the molecular requirements for sucrose transport with those for disaccharide sensing suggests that these sugars are perceived possibly at the plasma membrane level independently from sucrose transport.

Gibberellins (GA) induce *α-amylase* during the germination of barley (*Hordeum vulgare* L. cv Himalaya) grains (for review, see Fincher, 1989; Jacobsen et al., 1995; Bethke et al., 1997). Two tissues are sensitive to this hormone: the aleurone and the scutellar epithelium (Perata et al., 1997). As a consequence of α-amylase action on the starchy reserves, a large amount of soluble carbohydrates is produced, including hexoses and disaccharides. These soluble sugars strongly repress the action of gibberellic acid ($GA_3$) in the epithelium without affecting *α-amylase* expression in the aleurone (Perata et al., 1997).

The mechanisms of hormone perception have been subject of study for many years (for review, see Libbenga and Mennes, 1995), whereas sugar sensing is a relatively new subject of research. In recent years, a clearer understanding of the mechanisms involved in the perception of sugars as signaling molecules has been achieved (for review, see Graham, 1996; Koch, 1996; Jang and Sheen, 1997; Smeekens, 1998; Halford et al., 1999). The plant may sense a wide variety of sugars but, among soluble carbohydrates, hexoses and Suc are quantitatively predominant. The ability to sense these sugars has been demonstrated (Smeekens, 1998) and hexokinase may act as a hexose sensor in plants (Graham et al., 1994; Jang and Sheen, 1994; Jang et al., 1997). Beside hexoses, Suc may also act as a signaling molecule in plants (for review, see Smeekens and Rook, 1997; Lalonde et al., 1999), but data about the properties and identity of the putative Suc sensor are missing.

It is not known whether these sugar-signaling pathways act independently to trigger the modulation of distinct genes, or if they are part of an integrated sugar-signaling network.

In the present paper, we describe the existence of Glc and disaccharide-signaling mechanisms. We show that not only Glc, but also disaccharides are sensed through pathways leading to the modulation of *α-amylase* gene expression.

## RESULTS

### Both Glc and Suc Repress *α-amylase* Induction in Barley Embryos

*α-amylase* transcripts are absent in dry barley embryos; but transcription is induced by $GA_3$, and both Glc and Suc repress the action of $GA_3$ (Fig. 1A; Perata et al., 1997). Treatment with several concentrations of Glc and Suc indicates that both sugars similarly repress the induction of *α-amylase* (Fig. 1B). Glc and Suc are metabolically interconverted in barley embryos (data not shown; Fig. 2B) and the repression of *α-amylase* triggered by Glc and Suc can therefore be attributed to hexoses, Suc, or both.

### Other Carbohydrates Can Repress GA Signaling in Barley Embryos

We tested several disaccharides for their ability to repress the $GA_3$ induction of *α-amylase*, searching for carbohydrates able to trigger repression in the absence of metabolization into Glc, Fru, or Suc.

Glc, Fru, and Suc were, as expected, very effective in the repression of *α-amylase* induction, whereas mannitol used at the same concentration did not affect the induction of *α-amylase* (Fig. 2A). Repression

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was observed when the embryos were incubated in a solution containing the disaccharides palatinose, turanose, cellobiose, gentiobiose, lactulose, and leucrose (Fig. 2A). Melibiose was ineffective (Fig. 2A).

We tested whether the ability to repress *α-amylase* induction was attributable to the metabolism of dissacharides into Glc, Fru, or Suc. The data reported in Figure 2B show that treatment with a range of carbohydrates resulted in a wide variation in the sugar content of barley embryos. Leucrose, gentiobiose, and cellobiose were metabolized, as demonstrated by the significant increase in Glc, Fru, and Suc content.

To gain further insight about the possible metabolic utilization of the disaccharides under study, we investigated the effects of disaccharides on the growth and morphology of barley embryos. Barley embryos treated with metabolic sugars (Suc, Fru, and Glc) differ markedly from control embryos germinated in the absence of exogenous sugars, e.g. they show a more vigorous growth when compared to

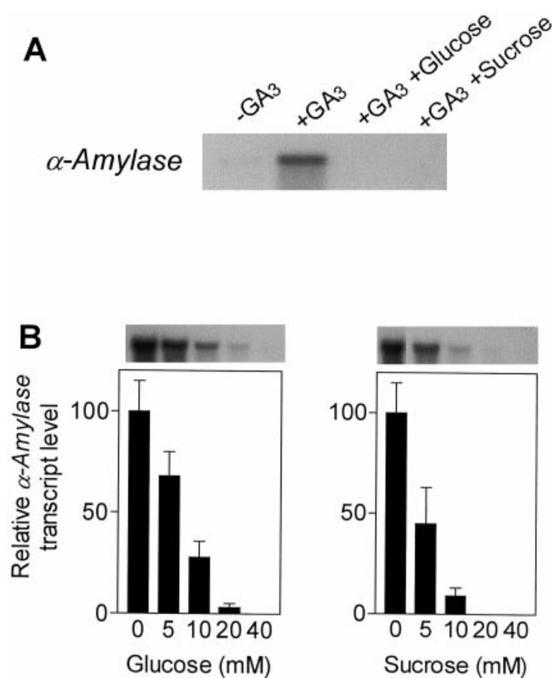


**Figure 1.** Glc and Suc repression of the induction of *α-amylase* in barley embryos. A, GA induction of *α-amylase* in barley embryos and repression by Glc and Suc. Embryos were incubated in 5 mM $CaCl_2$, 10 μM uniconazole, and, when used, 1 μM $GA_3$, 50 mM Glc, or 50 mM Suc for 24 h. Blots were probed with the *α-amylase* probe. B, Effect of Glc and Suc on the *α-amylase* mRNA level in barley embryos. Embryos were incubated for 24 h in 1 μM $GA_3$ and 5 mM $CaCl_2$ in the presence of increasing concentrations of Glc or Suc. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *α-amylase* probe. Membranes were reprobed with an rRNA probe (not shown). RNA levels of both *α-amylase* and rRNA were quantified and the relative *α-amylase* transcript level normalized to rRNA (100 = transcript level in the embryos not treated with exogenous sugars) is reported in the histograms. Data are means ± SE of three separate experiments. The mRNA band relative to 0 mM was duplicated in the two reproductions for the sake of clarity.

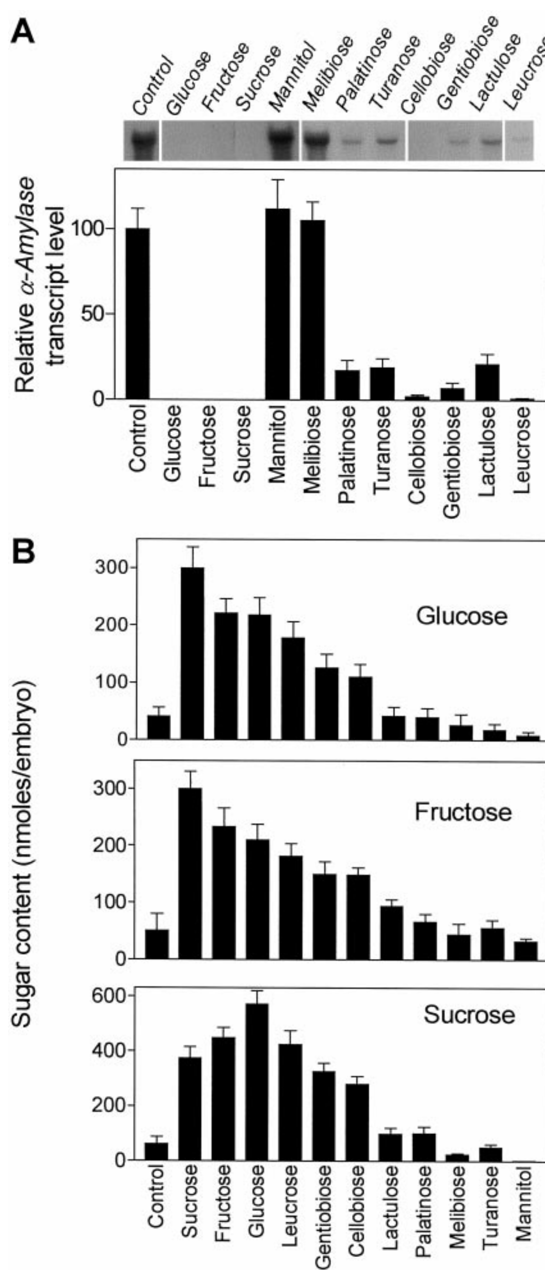


**Figure 2.** Screening of carbohydrates able to repress *α-amylase* induction in barley embryos. A, Effect of various carbohydrates on the *α-amylase* mRNA level in barley embryos. Embryos were incubated for 24 h in 1 μM $GA_3$ and 5 mM $CaCl_2$ in the presence of 100 mM carbohydrates. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *α-amylase* probe. Membranes were reprobed with an rRNA probe (not shown). RNA levels of both *α-amylase* and rRNA were quantified and the relative *α-amylase* transcript level normalized to rRNA (100 = Control) is reported in the histograms. Data are means ± SE of three separate experiments. The mRNA bands were rearranged in the reproduction for the sake of clarity. B, Glc, Fru, and Suc content in barley embryos fed with various carbohydrates. Embryos were incubated for 24 h in 1 μM $GA_3$ and 5 mM $CaCl_2$ in the presence of various carbohydrates (100 mM) and extracted for sugar assay. Data are means ± SE of four separate experiments.

that of control embryos (Fig. 3A, control and mannitol). Embryos fed with leucrose, gentiobiose, and cellobiose do not differ in their morphology from embryos treated with Glc, Fru, or Suc (Fig. 3A). On the contrary, embryos fed with lactulose, palatinose, melibiose, and turanose cannot be distinguished from the control embryos (Fig. 3A). These results suggest that these disaccharides are differently metabolized. Furthermore, feeding barley embryos with Suc, Fru, Glc, leucrose, gentiobiose, and cellobiose results in a dry weight that is doubled when compared to that of the control, whereas embryos treated with the other disaccharides show a dry weight not significantly different from that of the control (Fig. 3B). Overall, the results obtained indicate that lactulose, turanose, melibiose, and palatinose are not metabolized significantly when fed to barley embryos.

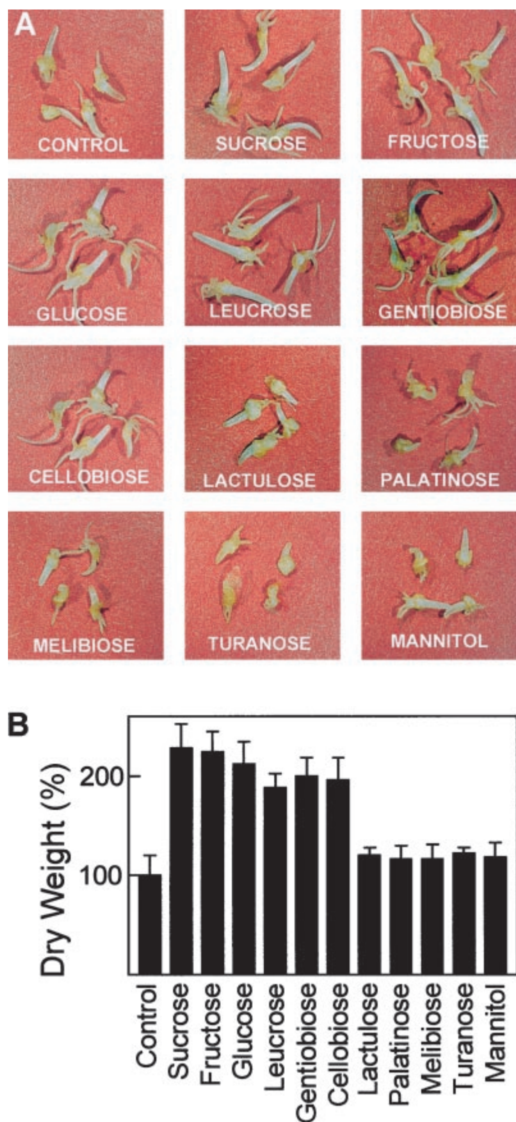


**Figure 3.** Effect of various carbohydrates on the germination of isolated barley embryos. A, Barley embryos were dissected from dry barley grains and incubated in 1 $\mu$M $GA_3$ and 5 mM $CaCl_2$ in the presence of 200 mM carbohydrates. After 48 h, the embryos were collected and photographed. A representative photograph is shown for each treatment. B, Dry weight of barley embryos (100 = Control) treated as described in A. Data are means ± SE of three replicates.

We tested whether the effects of disaccharides on *α-amylase* mRNA level are mediated by abscisic acid (ABA) or by effects on GA biosynthesis. ABA represses the induction of *α-amylase* and induces the *Rab16A* gene in barley embryos (Perata et al., 1997). Palatinose, turanose, lactulose (Figs. 2A and 7A), and ABA (Fig. 4A) repress *α-amylase*, but only the plant hormone induces the ABA-modulated *Rab16A* gene (Fig. 4B). The ABA content in embryos treated with these disaccharides does not differ from that of control embryos (data not shown; see Perata et al., 1997 for the ABA assay). It was previously shown that Glc repression of *α-amylase* is independent of effects on GA biosynthesis (Perata et al., 1997). This was confirmed for palatinose, which does not affect *α-amylase* induction by interfering with GA synthesis/perception, as demonstrated by its ability to repress *α-amylase* in embryos of the *slender* barley constitutive GA-response mutant (Fig. 4C).

### Glc Induces Transcription- and Protein Synthesis-Dependent *α-amylase* mRNA Destabilization

A sugar-induced reduction in the *α-amylase* mRNA level may be the result of either an inhibitory effect at the transcriptional level (Morita et al., 1998) or of an effect on *α-amylase* mRNA turnover. Experiments were performed with barley embryos pretreated with $GA_3$ for 12 h to induce *α-amylase.* Glc was added to the incubation media for an additional 8 h to observe its effect on mRNA level. Actinomycin D (ActD) was also used to evaluate the effects of sugars in the absence of transcriptional activity. Figure 5A shows that treatments with ActD prevented the increase of *α-amylase* mRNA level during the 8-h treatment, confirming the efficacy of this chemical in the inhibition of transcription. In the absence of transcription (Fig. 5A, +ActD), the *α-amylase* mRNA is stable. Addition of Glc in the absence of ActD remarkably reduced *α-amylase* mRNA stability, but Glc was ineffective in the presence of ActD (Fig. 5A, + Glc + ActD). The specificity of the effects observed was confirmed by the stability of the ubiquitin transcript (Fig. 5A). These results suggest that Glc affects *α-amylase* mRNA stability through a transcription-dependent mRNA destabilization process.

Protein synthesis is also needed for the Glc-induced *α-amylase* mRNA destabilization. Treating barley embryos with $GA_3$ for 12 h results in the induction of α-amylase (Fig. 5B, $GA_3$ 0–12 h). Prolonging the $GA_3$ treatment up to 20 h results in a higher transcript level (Fig. 5B, $GA_3$ 0–20 h). The α-amylase mRNA produced during the 0- to 12-h time interval is degraded if Glc is present during the 12- to 20-h interval (Fig. 5B, Glc 12–20 h). Addition of

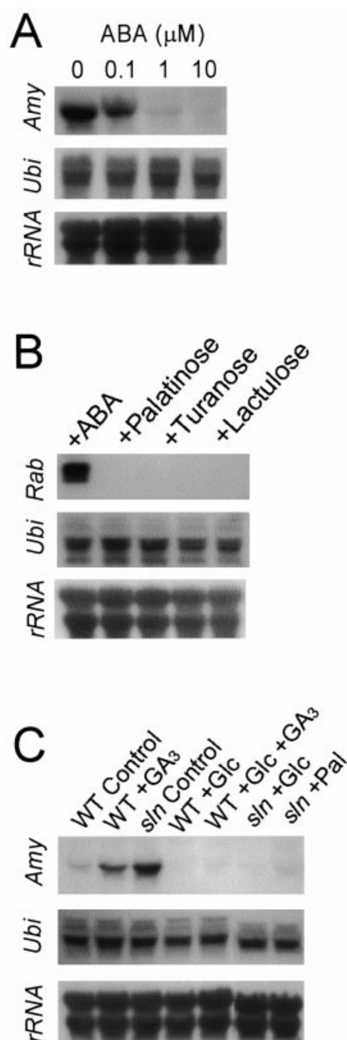


**Figure 4.** The disaccharide signaling is independent of ABA and of effects on gibberellin biosynthesis. A, Effect of ABA on *α-amylase* induction. Embryos were incubated for 24 h in 1 μM $GA_3$ and 5 mM $CaCl_2$ together with increasing ABA concentrations. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *α-amylase* probe. Membranes were reprobed with an rRNA probe as well as with an *ubiquitin* probe. B, Effect of disaccharides on the expression of the ABA-inducible *Rab16A* gene. Embryos were incubated for 24 h in 1 μM $GA_3$ and 5 mM $CaCl_2$. When present as a control, ABA was used at a concentration of 10 μM. Disaccharides were used at a concentration of 40 mM. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *Rab16A* probe. Membranes were reprobed with an rRNA probe as well as with an *ubiquitin* probe. C, Effect of Glc and palatinose on *α-amylase* induction at the mRNA level in embryos from the constitutive GA-response *slender* mutant. Both wild-type and *slender* embryos were treated with uniconazole, a GA-biosynthesis inhibitor (Izumi et al., 1984). Embryos were incubated in 5 mM $CaCl_2$, 10 μM uniconazole, and, when used, 1 μM $GA_3$, 40 mM Glc, or 40 mM palatinose for 24 h. Blots were probed with the α-amylase probe. Membranes were reprobed with an rRNA probe as well as with an ubiquitin probe. *Amy*, *α-amylase*; *Ubi*, *ubiquitin*; *Rab*, *Rab16A*; *sln*, *slender.*

the protein synthesis inhibitor cycloheximide (CHX) together with Glc (12–20 h) stabilizes the α-amylase mRNA (Fig. 5B).

### Disaccharides Differently Affect *α-amylase* mRNA Stability

To gain additional clues on the signaling pathways leading to *α-amylase* mRNA destabilization, we tested the effects of Suc, turanose, palatinose, and lactulose on the *α-amylase* mRNA level. Turanose palatinose, lactulose, Glc, and Suc repress *α-amylase* when fed to the barley embryos together with $GA_3$ at the beginning of the experiments, prior to *α-amylase* induction (Figs. 2 and 7). These sugars repress the $GA_3$-modulated induction of *α-amylase*. On the other hand, Glc destabilizes the otherwise very stable *α-amylase* mRNA (Fig. 5). In the experiments dealing with transcript stability (Fig. 5), sugars were added to embryos already expressing α-amylase (sugars added 12 h after the addition of $GA_3$; time 0 in Fig. 5 refers to sugars addition). As shown in Figure 6A, feeding Suc to barley embryos strongly decreased *α-amylase* mRNA stability, and ActD was able to prevent this effect. Turanose was unable to destabilize *α-amylase* mRNA (Fig. 6B), and comparable results were obtained using palatinose and lactulose (data not shown). Turanose does not affect the *α-amylase* mRNA stability (Fig. 6B) but, consistent with its effects on *α-amylase* expression reported in Figures 2 and 7, it represses any further increase in the *α-amylase* transcript level (compare 0 with 9 h in Fig. 6B, without ActD). A further increase is observed in the control experiment (Fig. 5A, control; compare 0 with 8 h).

Overall, the results indicate that turanose (as well other non-metabolizable disaccharides) represses *α-amylase* expression without affecting the stability of the transcript produced before its addition to the incubation medium. The effect of turanose is therefore distinct from those of Glc triggering *α-amylase* mRNA destabilization (Fig. 5A), as well as repression of the $GA_3$-mediated induction of *α-amylase* (Fig. 1).

### Structure-Function Relationships in Disaccharide Signaling in Barley Embryos

Lactulose, palatinose, and turanose are not metabolic sugars, but they repress the induction of *α-amylase* (Fig. 2). To gain further insight into the effects of these disaccharides, we performed experiments using RNA gel-blot analysis to identify the concentration threshold for repression of *α-amylase*. As shown in Figure 7A, 50% inhibition was obtained using 5 mM turanose, whereas slightly higher concentrations were needed to obtain a comparable repression when using palatinose and lactulose (Fig. 7A). The ubiquitin transcript was unaffected by the treatments (Fig. 7A).

The three disaccharides tested (Fig. 7A) possess a Fru moiety in their structure. We tested whether reduction of lactulose and palatinose, resulting in the disruption of the Fru moiety, alters the ability of these compounds to repress *α-amylase* induction. Lac-

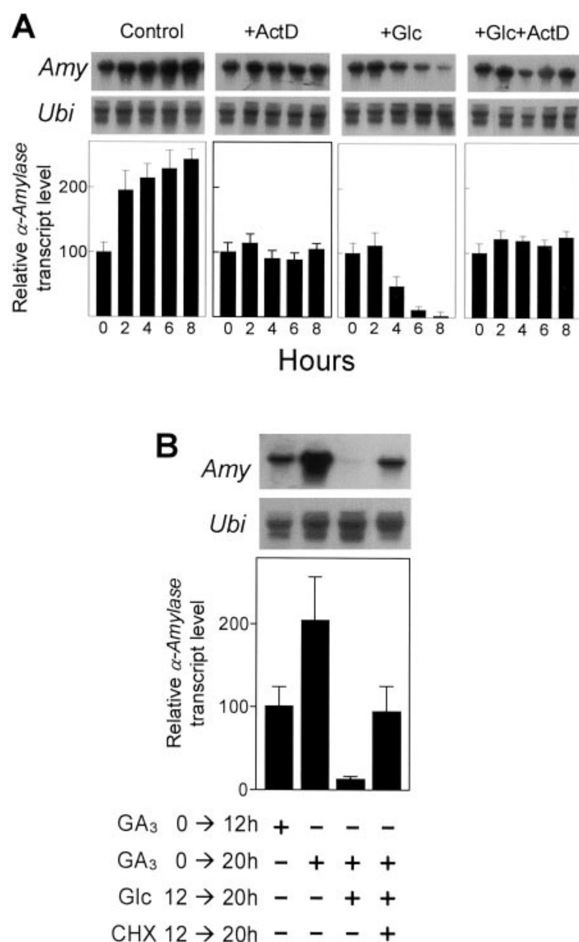


**Figure 5.** Glc triggers destabilization of the *α-amylase* mRNA through a transcription and protein synthesis-dependent pathway. A, Effect of ActD and Glc on α-amylase mRNA stability. ActD (10 μg/mL) and Glc (40 mM) were added to embryos pretreated for 12 h (time = 0) in 1 μM $GA_3$ and 5 mM $CaCl_2$; samples were collected every 2 h. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *α-amylase* probe. Membranes were reprobed with an rRNA probe (not shown) as well as with an *ubiquitin* probe. RNA levels of both *α-amylase* and rRNA were quantified and the relative *α-amylase* transcript level normalized to rRNA (100 = transcript level in the embryos pretreated with $GA_3$ for 12 h) is reported in the histograms. Data are means ± SE of three separate experiments. The mRNA band relative to 0 h was duplicated in the four reproductions for the sake of clarity. B, Effect of CHX and Glc, on the *α-amylase* mRNA stability. Embryos were pretreated for 12 h in 1 μM $GA_3$ and 5 mM $CaCl_2$; CHX (200 μM) and Glc (40 mM) were added to the embryos for an additional 8 h, up to a total time of 20 h. Presence of chemicals is as indicated by the + sign. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the α-amylase probe. Membranes were reprobed with an rRNA probe (not shown) as well as with an *ubiquitin* probe. RNA levels of both *α-amylase* and rRNA were quantified and the relative *α-amylase* transcript level normalized to rRNA (100 = transcript level in the embryos pretreated with $GA_3$ for 12 h) is reported in the histograms. Data are means ± SE of three separate experiments. *Amy*, *α-amylase*; *Ubi*, *ubiquitin*.

titol and palatinitol, reduced forms of lactulose and palatinose, respectively, do not repress *α-amylase* induction, even when used at 80 mM, suggesting that the intact fructosyl region is required for repression (Fig. 7B). Furthermore, replacing the Fru moiety of lactulose (β-Gal[1 → 4]Fru) with Glc (lactose, β-Gal[1 → 4]Glc) or Gal (4β-galactobiose, β-Gal[1 → 4]Gal) results in molecules unable to repress *α-amylase* (Fig. 7C). Melibiose (Gal[1 → 6]Glc; Fig. 2A), and 3α-galactobiose (Gal[1 → 3]Gal; Fig. 7C), devoid of a Fru moiety, are unable to repress *α-amylase* (Table I). All the Glc → Glc disaccharides tested, including nigerose and isomaltose (data not shown), represent a source of carbohydrates for barley embryo growth (Table I), and their effect on *α-amylase* repression cannot be distinguished from the effects of the hexoses resulting from their metabolization.

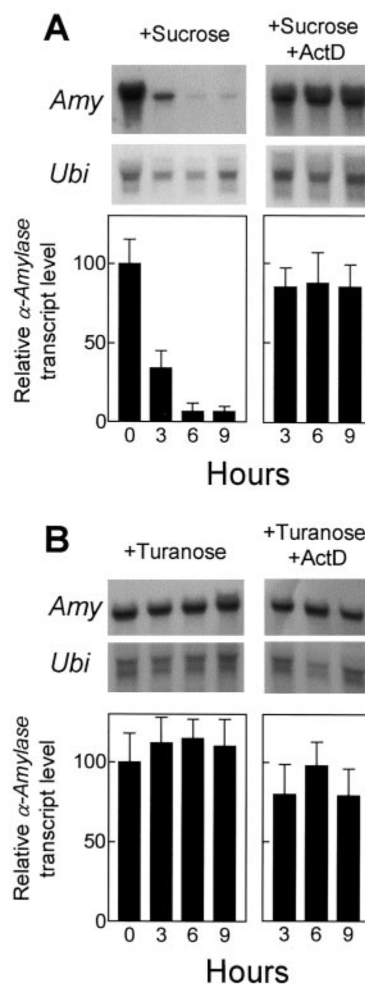


**Figure 6.** Suc but not turanose triggers destabilization of the *α-amylase* mRNA through a transcription dependent pathway. A, ActD (10 μg/mL) and Suc (40 mM) were added to embryos pretreated for 12 h (time = 0) in 1 μM $GA_3$ and 5 mM $CaCl_2$; samples were collected every 3 h. Embryos were extracted for RNA gel-blot analysis, performed as described in Figure 5. Data are means ± SE of three separate experiments. B, ActD (10 μg/mL) and turanose (40 mM) were added to embryos pretreated for 12 h (time = 0) in 1 μM $GA_3$ and 5 mM $CaCl_2$; samples were collected every 3 h. Embryos were extracted for RNA gel-blot analysis, performed as described in Figure 5. Data are means ± SE of three separate experiments. *Amy*, *α-amylase*; *Ubi*, *ubiquitin*.

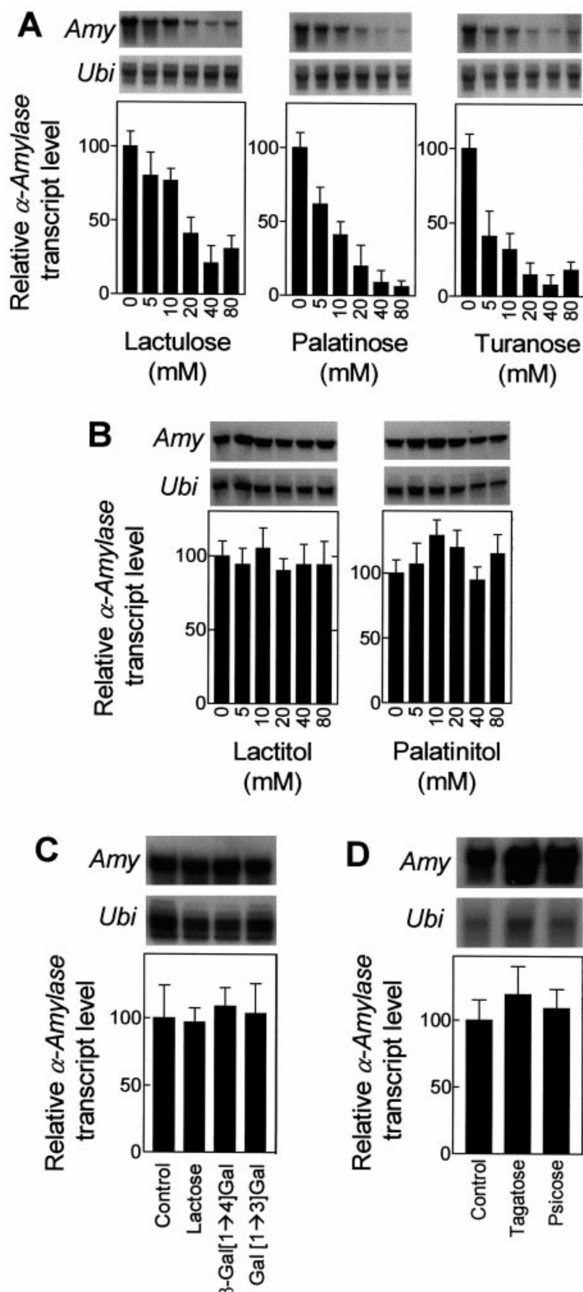


**Figure 7.** Effects of increasing concentrations of disaccharides on *α-amylase* induction in barley embryos. A and B, Effect of increasing concentration of lactulose, palatinose, turanose, lactitol, and palatinitol on the *α-amylase* mRNA level in barley embryos. Embryos were incubated for 24 h in 1 $\mu$M $GA_3$ and 5 mM $CaCl_2$ in the presence of 0 to 80 mM disaccharides. Embryos were extracted for RNA gel-blot analysis. Blots were probed with the *α-amylase* probe. Membranes were reprobed with an rRNA probe (not shown) as well as with an *ubiquitin* probe. RNA levels of both *α-amylase* and rRNA were quantified and the relative *α-amylase* transcript level normalized to rRNA (100 = transcript level in the embryos not treated with exogenous carbohydrates) is reported in the histograms. Data are means ± SE of three separate experiments. The mRNA band relative to 0 mM (lactulose, palatinose, and turanose) was duplicated in the three reproductions for the sake of clarity. C, Effect of lactose, galactobiose (β-Gal[1→4]Gal, and Gal[1→3]Gal) on the *α-amylase* mRNA level in barley embryos. Embryos treated as described in A and B with 80 mM carbohydrates. RNA blots were obtained as described in A and B. Data are means ± SE of three separate experiments. D, Effect of Fru epimers on the *α-amylase* mRNA level in barley embryos. Embryos treated as described in A and B with 80 mM tagatose or psicose. RNA blots were obtained as described in A and B. *Amy*, *α-amylase*; *Ubi*, *ubiquitin*.

The Fru moiety of lactulose/palatinose/turanose is linked to Gal/Glc/Glc through position 4/6/3 respectively (Table I), suggesting that positions 4/6/3 of the Fru moiety do not play an important role in the molecular recognition of the disaccharides. We tested if C4 and C3 epimers of Fru (tagatose and psicose) could repress *α-amylase.* The results indicate that neither tagatose nor psicose repress *α-amylase* induction (Fig. 7D).

## DISCUSSION

The rapid metabolization of Suc into its constituent hexoses hampers easy approaches to Suc sensing. The same applies to hexose sensing, since most plant tissues can readily synthesize Suc when fed with hexoses. An exception to this rule is given in the experiments dealing with the effect of Suc on genes whose expression is not affected by hexoses. Suc sensing has been demonstrated for the modulation of the patatin promoter (Wenzler et al., 1989; Jefferson et al., 1990), of the *rolC* promoter in transgenic tobacco (Yokoyama et al., 1994), and of the proton-Suc symporter activity in sugar beet (Chiou and Bush, 1998). Furthermore, Suc represses translation of a transcription factor in Arabidopsis (Rook et al., 1998). In these experiments, the authors could separate the effects of Suc from those related to its metabolism into Glc and Fru, since the effect of these hexoses was either absent or less pronounced when compared to those of Suc.

Our experiments show that both Suc and Glc affect the expression of *α-amylase* in barley embryos and that these sugars are rapidly interconverted. We used a series of disaccharides to establish the ability of barley embryos to sense disaccharides, to discriminate from their effect and that of Glc, and to gain clues about the disaccharide-sensing machinery.

### The Existence of Disaccharide Sensing Is Emphasized by the Use of Non-Metabolic Sugars

The use of non-metabolic sugars is a useful tool for investigating sugar sensing, but it also requires a series of investigations aimed at establishing their possible metabolism and toxicity. Indeed, the widely used Glc analog 2-D-Glc shows toxic effects on plant systems (Graham et al., 1994), and recent experimental data provided evidence of its metabolization into 2-deoxy-Suc (Klein and Stitt, 1998).

**Table I.** *Metabolization and ability to repress α-amylase induction of the disaccharides used in this study*

Metabolism is defined as the ability of the disaccharides to induce an increase in the endogenous content of Glc+Fru+Suc equal to or exceeding two times that of control, as well as in bringing about a significant (two times that of control) increase in the dry wt of the isolated embryos. Data on *α-amylase* repression are based on the ability of the tested disaccharides (80 mM) to repress *α-amylase* induction by at least 70%.

| Compound | Chemical Structure | Metabolized | *α-amylase* Repression |
|---|---|---|---|
| Suc | Glc[1→2]Fru | Yes | Yes |
| Turanose | Glc[1→3]Fru | No | Yes |
| Nigerose | Glc[1→3]Glc | Yes | Yes |
| 3α-Galactobiose | Gal[1→3]Gal | No | No |
| Cellobiose | β-Glc[1→4]Glc | Yes | Yes |
| Lactulose | β-Gal[1→4]Fru | No | Yes |
| Lactitol | β-Gal[1→4]Glucitol | No | No |
| Lactose | β-Gal[1→4]Glc | No | No |
| 4β-Galactobiose | β-Gal[1→4]Gal | No | No |
| Leucrose | Glc[1→5]Fru | Yes | Yes |
| Isomaltose | Glc[1→6]Glc | Yes | Yes |
| Gentiobiose | β-Glc[1→6]Glc | Yes | Yes |
| Palatinose | Glc[1→6]Fru | No | Yes |
| Palatinitol | Glc[1→6]Glucitol (50%)<br>Glc[1→6]Mannitol (50%) | No | No |
| Melibiose | Gal[1→6]Glc | No | No |

The compounds tested in this study are not toxic, because they do not affect the germination of barley embryos. Furthermore, feeding palatinose, lactulose, and turanose to barley embryos does not negatively affect $^{14}CO_2$ production from [$^{14}$C]Suc or [$^{14}$C]Glc (data not shown). These disaccharides affect the GA signaling independently of ABA (Fig. 4). Furthermore, they affect α-amylase expression downstream of the *slender* mutation and thus independently from effect(s) on GA synthesis or perception.

The effects of palatinose, turanose, and lactulose are independent of their metabolism into constituent hexoses. This statement is supported by the following experimental evidence: (a) These disaccharides are not significantly metabolized into Glc, Fru, or Suc, but are as effective as Glc or Suc in repressing *α-amylase* (compare with Figs. 1B and 7A); (b) they do not enhance the growth of barley embryos (e.g. embryo morphology mirrors that of control, sugar-starved embryos; Fig. 3A); (c) the dry weight of barley embryos treated with the above cited disaccharides does not differ from that of control embryos (Fig. 3B); and (d) these disaccharides do not destabilize *α-amylase* mRNA (Fig. 6B). The first three pieces of experimental evidence are of interest but not conclusive, since the sugar content/metabolism pattern in the whole embryo may not reflect the actual hexose concentration/metabolism in the different tissues present in the embryo. The latter evidence reflects more accurately the actual hexose concentration in the scutellar epithelium expressing *α-amylase*, since metabolism into hexoses would have had consequences on the *α-amylase* mRNA stability.

### *α-amylase* mRNA Destabilization Highlights the Existence of Distinct Glc and Disaccharide Sensing

The *α-amylase* transcript is destabilized through a mechanism requiring de novo Glc-induced transcription. The effect of ActD is somewhat surprising. It is known that sugar starvation results in an increased *α-amylase* mRNA half-life in rice cells (Sheu et al., 1996; Chan and Yu, 1998), but ActD is unable to prevent Glc effects in rice suspension cultures (Sheu et al., 1996). De novo protein synthesis is needed for *α-amylase* mRNA destabilization in rice suspension cultures (Sheu et al., 1994), in agreement with our results obtained using CHX.

Suc, as well as all the disaccharides tested (not shown) that are metabolically broken-down into their constituent hexoses, represses *α-amylase* induction (Fig. 1B) and also induces destabilization of *α-amylase* mRNA (Fig. 6A). Therefore, we could not separate the effects of the hexoses derived from the metabolism of disaccharides from the effects due to their possible direct sensing. On the contrary, turanose, palatinose, and lactulose do not affect *α-amylase* mRNA stability (Fig. 6B; data not shown). These disaccharides, triggering an effective repression of *α-amylase* induction (Fig. 7A), are therefore sensed through a sensing machinery distinct from the one responsible for mRNA destabilization.

### Structure-Function Relationships in Disaccharide Sensing in Barley Embryos

Lactulose, palatinose, and turanose possess a Fru moiety but they differ from one another for the other

moiety (Gal, Glc, and Glc, respectively), as well as for the chemical link position (1→4, 1→6, and 1→3, respectively). This is suggestive of a possible Fru-specific recognition of these molecules. Supporting this view, we found that reducing the Fru moiety of lactulose ($\beta$-Gal[1→4]Fru) and palatinose (Glc[1→6] Fru) results in molecules (lactitol and palatinitol) unable to repress *α-amylase* induction (Fig. 7B). Furthermore, lactose ($\beta$-Gal[1→4]Glc) as well as 4$\beta$-galactobiose ($\beta$-Gal[1→4]Gal) are unable to repress *α-amylase* (Fig. 7C), reinforcing the evidence that suggests that the fructosyl region of lactulose is needed for repression. The other non-metabolizable disaccharides that were unable to repress *α-amylase* are devoid of a Fru moiety, i.e. melibiose (Gal[1→6]Glc; Fig. 2A) and 3$\alpha$-galactobiose (Gal[1→3]Gal; Fig. 7C). However, although an intact Fru moiety is required for *α-amylase* repression (compare with Fig. 7, A–C), Fru epimers (C3 and C4) are ineffective (Fig. 7D), suggesting that the Fru moiety should be part of a disaccharide to trigger repression, and/or that the steric position of hydrogen at positions 3 and 4 in the Fru molecule is important for recognition. The presence of the free hydroxyl groups of Fru at positions C3, C4, and C6 is not required, however, as indicated by the equal efficacy of lactulose, palatinose, and turanose in which the OH group at positions C3, C4, and C6 of the Fru moiety is involved in the link with the aldohexose.

Although our experiments with palatinose and turanose (two Suc analogs) do not represent direct evidence for Suc sensing in barley embryos, this possibility is likely. Indeed, specific Suc sensing has been demonstrated in various plant systems (Wenzler et al., 1989; Jefferson et al., 1990; Yokoyama et al., 1994; Chiou and Bush, 1998; Rook et al., 1998) and the possible involvement of a Suc transporter as part of the Suc sensing machinery has been discussed, which highlights the lack of direct evidence supporting or disproving this hypothesis (Smeekens and Rook, 1997). Our data do not provide evidence for an involvement of a Suc transporter in disaccharide sensing. Palatinose, turanose, and lactulose are not recognized by Suc transporter(s) and do not compete for Suc transport (Schmitt et al., 1984; M'Batchi and Delrot, 1988; Li et al., 1994), but they repress *α-amylase* induction. Structure-function data suggest that the fructosyl region is needed for *α-amylase* repression (this study). The fructosyl unit is required for a hydrophobic interaction between Suc and its transporter but the hydroxyl groups on the Glc residue are responsible for substrate specificity (Hecht et al., 1992; Bush, 1993). Indeed, reversing the orientation of the hydroxyl group at position C4 of Glc derivatives decreases the competitive inhibition of Suc transport (Hecht et al., 1992), whereas a Gal-containing disaccharide is as effective as Glc-containing disaccharides in *α-amylase* repression, granted a Fru moiety is linked to the aldohexose.

Interestingly, palatinose fed to plant protoplasts does not induce membrane depolarization, indicating the absence of an $H^+$-sugar symport system able to transport this disaccharide into the plant cell (Bouteau et al., 1999). Even though the existence of an intracellular disaccharide sensor cannot be ruled out, it is tempting to speculate that palatinose is possibly sensed at the plasma membrane level. This possibility would be in agreement with the proposal of sensors evolved from transporters (Lalonde et al., 1999), in analogy with the yeast monosaccharide sensors showing homology with Glc permeases but unable to transport Glc.

Advancement in the knowledge about the Suc transporter gene family and the possible existence of sugar sensing at the plasma membrane uncoupled from transport will likely lead to a deeper understanding about sugar sensing in plants.

## MATERIALS AND METHODS

### Plant Material

Barley (*Hordeum vulgare* L. cv Himalaya) grains (1995 harvest, Washington State University, Pullman, WA) were used. Embryos were dissected from sterilized grains (shaken in 5% [w/v] sodium hypochlorite for 1 h and washed in sterile water with shaking for 2 h) using a scalpel. Only intact embryos with no starch or aleurone tissue adhering to the scutellar tissue were used. Incubation of embryos was carried out in 24-well plastic plates, each well containing four embryos and 500 $\mu$L of 5 mM $CaCl_2$ containing 5 $\mu$g of chloramphenicol. Embryos were incubated at 25°C with vigorous shaking. When used, 1 $\mu$M $GA_3$, 10 $\mu$M ABA, and 10 $\mu$M uniconazole [(*E*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol; Sumitomo Chemical Co., Takarazuka, Japan] were added.

### Chemicals

The commercially available compounds were purchased from Sigma (St. Louis). Disaccharides used in this study were tested for their possible contamination with Glc, Fru, or Suc, and this lead us to exclude maltulose from further testing, since the commercial preparation was found to be contaminated with Glc and Fru. The other compounds were found to be free from contaminating sugars.

### *slender* Barley Embryo Identification

We used embryos isolated from the *slender* mutant of barley, a constitutive GA-response mutant (Chandler, 1988; Lanahan and Ho, 1988) having the GA perception-signal transduction pathway constitutively activated (Hooley, 1994) and whose phenotype is not influenced by GA biosynthesis inhibitors (Croker et al., 1990). Barley grains with *slender* mutants in a cv Himalaya background were obtained from M. Robertson (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia). The *slender* mutant is self-sterile and must be maintained as

a heterozygous population. Grains from the heterozygous plants segregate into three wild type and one *slender*. Mutant grains were identified by the starch plate method described by Lanahan and Ho (1988). Half-grains were tested, whereas the corresponding embryos were stored at 4°C. After being identified as wither wild-type or *slender* mutants, the embryos were used for the experiments.

### Assay of Carbohydrates

Samples (0.1–0.5 g fresh weight) were rapidly frozen in liquid nitrogen and ground to a powder, extracted as described by Tobias et al. (1992), and assayed through coupled enzymatic assay methods, measuring the increase in $A_{340}$. The efficiency of the method was tested by using known amounts of carbohydrates. Incubation of the samples and standards were carried out at 37°C for 30 min. The reaction mixture (1 mL) was as follows: Glc, 100 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM ATP, 0.6 mM NADP, 1 unit hexokinase, and 1 unit of Glc-6-P dehydrogenase; Fru was assayed as described for Glc with the addition of 2 units of phosphoglucoisomerase; the increase in $A_{340}$ was recorded. Suc was first hydrolyzed using 85 units of invertase (in 15 mM sodium acetate, pH 4.6) and the resulting Glc and Fru were assayed as described above. The carbohydrates used in this study did not interfere with the sugar assays.

### cDNA Probes

The high-pI $\alpha$-amylase probe was clone pM/C (Rogers, 1985); the probe for detecting the ABA-inducible *Rab* gene was *Rab16A* (Mundy and Chua, 1988). The probe for rRNA was a rice rRNA probe, and the ubiquitin probe was a barley probe detecting different size messengers of the ubiquitin multigene family (Gausing and Barkardottir, 1986).

### RNA Isolation and Gel Blots

RNA extraction was performed by using the aurintricarboxylic acid method as previously described (Perata et al., 1997). The amount of total RNA loaded in electrophoresis was 20 $\mu$g. RNA was electrophoresed on 1% (w/v) agarose-formaldehyde gels, and blotted on nylon membrane (BrightStar-Plus, Ambion, Austin, TX) by using the procedure suggested by the manufacturer. Membranes were prehybridized and hybridized using the NorthernMax kit (Ambion). Radiolabeled probes were prepared from gel-purified cDNA inserts by random primer labeling (Takara Chemicals, Tokyo) with [$\alpha$-$^{32}$P]dCTP. Equal loading was checked by reprobing with an rRNA and ubiquitin cDNA probe. RNA was quantified after image acquisition using a digital camera and the Band Leader software (Magnitec, Tel-Aviv). Statistical significance of the data reported in the RNA gel blots was checked by analyzing at least three replicate experiments and their quantitative, rRNA-normalized data after image acquisition.

## NOTE ADDED IN PROOF

In an interesting recent review article, Sonnewald and Herbers (1999) claimed that palatinose and turanose repress the *rbcS* gene and induce the *PR-Q* transcripts in tobacco leaves.

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