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BRIEF COMMUNICATION

The fructose transport mechanism in microsomal membrane vesicles from rye roots

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Abstract

In a fructose-containing medium in which rye root-microsomal membrane vesicles had reached the equilibrium of uptake of fructose, the presence of both Mg^{2+} and ATP caused the efflux of fructose from the vesicles. Among nucleotides examined, ATP caused the largest efflux of fructose. The efflux of fructose dependent on Mg^{2+} and ATP was quite insensitive to a protonophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which actually abolished MgATP-dependent proton accumulation in the vesicles, while it was largely inhibited by vanadate, which inhibits ATP-binding cassette transporters (ABCTs). The Michaelis-Menten constant (Km) of the efflux of fructose was 0.4 mM. It was observed that fructose stimulated the ATPase activity of the vesicles and that vanadate markedly decreased the fructose-stimulated ATPase activity.

Additional key words: ATP-binding cassette transporter, MgATP, ΔpH, *Secale cereale*.

Sucrose is a major photosynthetic product and is a major translocation substance in most higher plants. Significant amounts of sucrose in higher plant cells are hydrolyzed to glucose and fructose by invertases existing in the cell wall, cytosol and vacuole (Sturm and Tang 1999). The mechanisms of the facilitated diffusion of these monosaccharides at the plasma membrane and vacuolar membrane and the proton pump-dependent, ΔpH-driven co-transport of each monosaccharide with proton at the plasma membrane have been found (Stanzel *et al.* 1988, Milner *et al.* 1995, Buckhout and Tubbe 1996, Shiratake *et al.* 1997, Büttner and Sauer 2000). Recently, in a microbe, *Sinorhizobium meliloti*, uptake of fructose has been demonstrated to be mediated by an ATP-binding cassette transporter (ABCT) (Lambert *et al.* 2001), however, whether such ABCT of fructose is present in the membrane system of higher plants is not clear. Microsomal membrane vesicles prepared from higher plants are a powerful tool for identifying transporters in the membrane system (Sze 1985). Thus, in the present study, using microsomal membrane vesicles from rye roots we examined whether such ABCT of fructose is present in the vesicles. The experiments were mainly carried out using microsomal membrane vesicles that had reached the equilibrium of uptake of fructose in a fructose-containing medium.

Roots (fresh mass, 40 g) of rye (*Secale cereale* L. cv. Elbon) plants grown for 11 d in a 1/10 Hoagland solution under conditions described previously (Kasai *et al.* 1998) were homogenized with a polytron (Model *PCU-11*, *Kinematica AG*, Lucerne, Switzerland) in a solution (200 cm^3) containing 50 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES)-1,3-bis[tris (hydroxy-methyl)methylamino]-propane (BTP) (pH 7.6), 5 mM EDTA-BTP (pH 7.6), 5 mM EGTA-BTP (pH 7.6), 10 mM dithiothreitol (DTT), 2.5 mM $Na_2S_2O_5$, 0.5 % (m/v) bovine serum albumin (BSA), 2 mM phenyl-methylsulfonyl fluoride (PMSF) and 1.5 % (m/v) polyvinyl polypyrrolidone (P-6755, *Sigma*, St. Louis, USA) on ice, and the homogenate was filtered through

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Abbreviations: ABCT(s) - ATP-binding cassette transporter(s); BSA - bovine serum albumin; BTP - 1,3-bis[tris(hydroxymethyl)-methylamino]-propane; CCCP - carbonylcyanide *m*-chlorophenylhydrazone; DTT - dithiothreitol; HEPES - *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; Km - Michaelis-Menten constant; PMSF - phenylmethylsulphonyl fluoride.

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four layers of gauze. The filtrate was centrifuged at 10 000 *g* for 20 min at 4 °C, and the supernatant was centrifuged at 80 000 *g* for 40 min at 4 °C. The pellet was suspended in a solution (80 cm^3) containing 50 mM HEPES-BTP (pH 7.0), 0.1 % (m/v) BSA and 1 mM DTT, and again centrifuged at 80 000 *g* for 40 min at 4 °C. The obtained pellet was suspended in the same solution (6 cm^3), divided into small aliquots and stored at -80 °C. The suspensions were thawed and used as the sample of microsomal membrane vesicles.

Fructose transport across the microsomal membrane vesicles was assayed by a gel-filtration method (Shiratake *et al.* 1997). Unless otherwise stated, vesicles (protein, 150-180 µg) were incubated for 30 min at 25 °C in a 1 mM fructose-containing medium (50 mM HEPES-BTP (pH 7.0), 0.1 % (m/v) BSA, 1 mM DTT, 1 mM EDTA-BTP (pH 7.0), total volume, 0.1 cm^3). Subsequently, a fructose-containing medium (0.02 cm^3) that contained $MgSO_4$ or ATP-BTP (pH 7.0) or both or, as a control, the fructose-containing medium (0.02 cm^3), was added. The final concentrations of $MgSO_4$ and ATP were 6 and 5 mM, respectively. After incubation for 1 min, the medium (0.1 cm^3) was applied to a gel (*Sephadex G-50*, 0.175 g in dry mass)-packed column pre-equilibrated with a solution containing 50 mM HEPES-BTP (pH 7.0), 0.1 % (m/v) BSA, 1 mM EDTA-BTP (pH 7.0) and 1 mM DTT, and then, the column was centrifuged at 100 *g* for 2 min. The eluate (almost 0.1 cm^3) from the column, which contained the vesicles, was dropped into a solution containing Triton X-100 (final concentration 0.75 %, m/v) and EDTA-BTP (pH 7.0) (final concentration 10 mM), which had been added at the bottom of a plastic tube supporting the column. This mixture was used to determine the amount of fructose within the vesicles. The determination was made at 25 °C in a coupled reaction with hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase by monitoring the reduction of $NADP^+$ (Stitt *et al.* 1989) with a fluorometer (*F-2000, Hitachi,* Tokyo, Japan; excitation 360 nm, emission 460 nm). The assay medium (total 2 cm^3) contained 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.02 % (m/v) Triton X-100, 2.5 mM ATP, 0.33 mM $NADP^+$, 1 U hexokinase, 1 U phosphoglucose isomerase, 1 U glucose-6-phosphate dehydrogenase and the mixture. The relationship between different amounts of standard fructose and fluorescent intensity of NADPH generated as a result of the enzymatic reaction was linear ($r^2 > 0.99$) in the range of the analyzed amounts of fructose within the vesicles in the transport experiments.

The MgATP-dependent proton transport into the vesicles and ATPase activity associated with the vesicles were examined at 25 °C in similar media as used for the fructose transport assay, respectively, by monitoring the fluorescence quenching of quinacrine with a fluorometer (*F-2000*; excitation 420 nm, emission 495 nm) for the former and by quantifying the phosphate released from ATP according to the method of Heinonen and Lahti (1981) for the latter. The medium for the former assay (total 2 cm^3) contained 50 mM HEPES-BTP (pH 7.0), 0.1 % (m/v) BSA, 1 mM DTT, 1 mM EDTA-BTP (pH 7.0), 3 µM quinacrine, 6 mM $MgSO_4$, 5 mM ATP-BTP (pH 7.0), 0 or 100 mM KCl and the vesicles (protein, 100 µg). The medium for the latter assay (total 0.5 cm^3) contained 50 mM HEPES-BTP (pH 7.0), 0.1 % (m/v) BSA, 1 mM DTT, 1 mM EDTA-BTP (pH 7.0), 6 mM $MgSO_4$, 5 mM ATP-BTP (pH 7.0), 0.02 % (m/v) Triton X-100, 0 or 2 mM fructose and the vesicles (protein, 10 µg). Protein content was quantified by the method of Bradford (1976) using BSA as a standard.

During the incubation of the vesicles in the fructose-containing medium, vesicles took up fructose from the medium and after 30 min almost reached the equilibrium of uptake of fructose (Fig. 1*A*). Subsequent 1 min-incubation of the vesicles with $MgSO_4$ or ATP in the fructose-containing medium did not significantly affect the amount of fructose within the vesicles (Fig. 1*B*). Subsequent 1 min-incubation of the vesicles with BTP-SO_4 and ATP also did not significantly affect the amount of fructose within the vesicles (not shown). On the other hand, the incubation with both $MgSO_4$ and ATP significantly decreased the amount of fructose within the vesicles (Fig. 1*B*). These results indicate that Mg^{2+} and ATP caused the efflux of fructose from the vesicles. Among the nucleotides examined (ATP, GTP, UTP and ADP), ATP gave the highest rate of efflux of fructose (Fig. 2*A*).

There is a possibility that the observed Mg^{2+}, ATP-dependent efflux of fructose from the vesicles might be caused by the proton pump-dependent, ΔpH-driven proton/monosaccharide co-transport. Usually, significant proton pumping activity of microsomal membrane vesicles from higher plants is seen in the presence of very permeant anions such as Cl^-, because the anions with a negative electrical charge strongly repress the development of membrane potential resulting from proton transport (Sze 1985). The microsomal membrane vesicles prepared from rye roots showed MgATP-dependent proton accumulation in the presence of KCl (100 mM) in a similar medium to the fructose-containing medium. The addition of a protonophore, CCCP (15 µM), to the medium abolished the proton accumulation in the vesicles. On the other hand, much reduced MgATP-dependent proton accumulation in the vesicles was observed in the absence of KCl (see inset in Fig. 2*A*). Since the fructose-containing medium did not contain any readily permeant anions, the above-mentioned possibility is unlikely. In fact, Mg^{2+}, ATP-dependent efflux of fructose from the vesicles was not significantly affected by the presence of CCCP (15 µM) (Fig. 2*A*), indicating that proton pump-dependent, ΔpH-driven proton/monosaccharide cotransport was not the cause of the efflux of fructose. Vanadate inhibits ABCTs (Martinoia *et al.* 2002). Mg^{2+}, ATP-dependent efflux of fructose from the vesicles was markedly inhibited in the presence of vanadate (Na_3VO_4, 0.5 mM) (Fig. 2*A*).

The microsomal membrane vesicles from rye roots reached the equilibrium of uptake of fructose when incubated for 30 min not only in 1 mM fructose-containing medium but also in 0.5, 2 or 4 mM fructose-containing medium, and the amount of fructose within the vesicles at the end of the incubation was observed to be proportional to the concentration of fructose in fructose-containing medium in which vesicles were incubated (not shown). Subsequent 1 min-incubation of the vesicles with $MgSO_4$ and ATP in each fructose-containing medium caused the efflux of fructose from the vesicles, and when the rates of the Mg^{2+}, ATP-dependent efflux of fructose were plotted against the concentration of fructose in the fructose-containing medium in which vesicles were incubated, they were shown to represent a profile of the enzymatic kinetics (Fig. 2*B*). The Hanes plot-analysis of the data showed that the Km (fructose) value was 0.4 mM, which is within the range of the physiological concentrations of fructose in higher plant cells.

In the presence or absence of fructose at 2 mM, at which an almost maximal rate of Mg^{2+}, ATP-dependent efflux of fructose was obtained, we determined the ATPase activity associated with the vesicles. As a result, fructose was shown to stimulate the ATPase activity. The fructose-stimulated ATPase activity was 804 ± 134 nmol(Pi) mg^{-1}(protein) h^{-1} (n = 4). The presence of vanadate (Na_3VO_4, 0.5 mM), which had an inhibitory effect on the Mg^{2+}, ATP-dependent efflux of fructose from the vesicles, largely decreased the fructose-stimulated ATPase activity. The fructose-stimulated ATPase activity in the presence of vanadate was 236 ± 68 nmol(Pi) mg^{-1}(protein) h^{-1}.

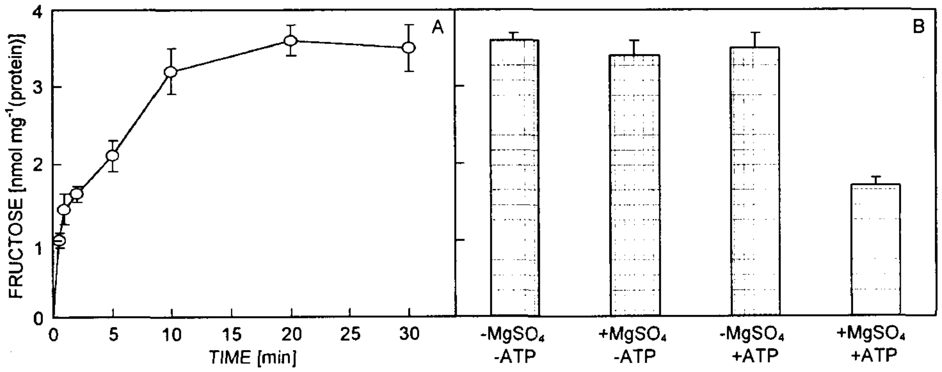

Fig. 1. *A* - Increase in fructose content within rye root-microsomal membrane vesicles during 30 min-incubation in 1 mM fructose-containing medium. *B* - The content of fructose within rye root-microsomal membrane vesicles after subsequent 1 min-incubation without $MgSO_4$ and ATP or with $MgSO_4$ and/or ATP in 1 mM fructose-containing medium. *Vertical bars* denote SD (n = 4).

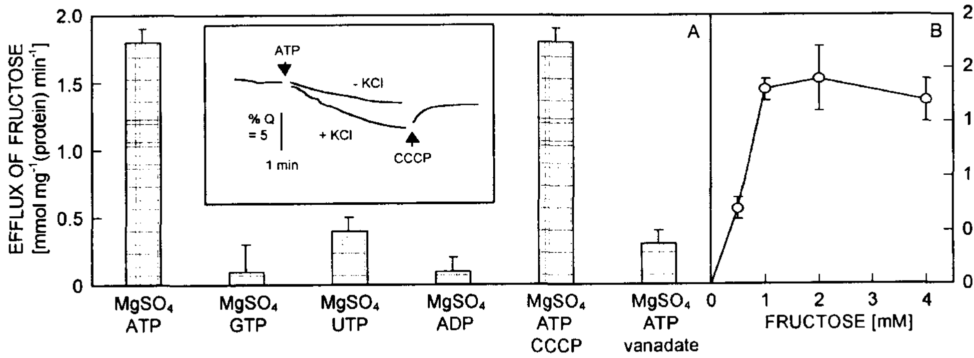

Fig. 2. *A* - Efflux of fructose from rye root-microsomal membrane vesicles caused by 1 min-incubation with $MgSO_4$ and ATP, with $MgSO_4$ and other nucleotides, or with $MgSO_4$ and ATP together with CCCP (15 μM) or vanadate (Na_3VO_4, 0.5 mM) in 1 mM fructose-containing medium. The vesicles were incubated for 30 min in the fructose-containing medium and then subjected to the incubation. The efflux was calculated by subtracting the amount of fructose within the vesicles analyzed after 1 min-incubation with the substances indicated from that analyzed after 1 min-incubation without these additions. MgATP-dependent proton transport into the vesicles determined in the presence or absence of KCl (100 mM) in a similar medium to the fructose-containing medium and the effect of CCCP (15 μM) on the proton transport are shown in the inset. The reaction was started by the addition of ATP in the presence of $MgSO_4$. Q: fluorescence quenching of quinacrine. *B* - Efflux of fructose from rye root-microsomal membrane vesicles caused by 1 min-incubation with $MgSO_4$ and ATP in fructose-containing medium. The vesicles were incubated for 30 min in each fructose-containing medium and then subjected to the incubation. The efflux was calculated as described in *A*. *Vertical bars* denote SD (n = 4).

The well-known higher plant monosaccharide transporter is a proton pump-dependent, ΔpH-driven proton/monosaccharide co-transporter, STP-1 (Büttner and Sauer 2000). This transporter mediates glucose transport preferably over fructose transport (Boorer *et al.* 1994). An energy-dependent transporter that preferably transports fructose is not yet known to be present in higher plants. We also analyzed the Mg^{2+}, ATP-dependent efflux of glucose from rye root-microsomal membrane vesicles in a similar way as for fructose. The amount of glucose within the vesicles also was determined enzymatically, but without using phosphoglucose isomerase (Stitt *et al.* 1989). The profile of uptake of glucose by the vesicles during 30 min-incubation in a glucose (1 mM)-containing medium was almost similar to that obtained for fructose. However, the rate of Mg^{2+}, ATP-dependent efflux of glucose, which was analyzed as in the case of fructose, was significantly smaller than that of fructose: the rates for glucose and fructose were 0.5 ± 0.1 and 1.8 ± 0.1 nmol mg^{-1}(protein) min^{-1} ($n = 4$), respectively (the latter was cited from Fig. 2*A*).

The Mg^{2+}, ATP-dependent efflux of fructose from rye root-microsomal membrane vesicles was ΔpH-independent and inhibited by vanadate, and fructose-stimulated ATPase activity sensitive to vanadate was found in the vesicles. These facts suggest that the efflux of fructose may be mediated by ABCT. A recent study has demonstrated that *Sinorhizobium meliloti*, a microbe, has an ABCT for fructose (Lambert *et al.* 2001). Comparative analysis at the gene level may provide valuable information. Nevertheless, our data at least demonstrate that a ΔpH-independent and Mg^{2+}, ATP-dependent fructose transport mechanism is present in the microsomal membrane vesicles from rye roots.

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