Planta (1989) 179:403–408

Planta

D-Mannose uptake by fenugreek cotyledons

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Abstract. The uptake of D-mannose was studied in detached cotyledons of germinated fenugreek (*Trigonella foenum-graecum* L.) seeds. Uptake kinetics indicate the involvement of two components, a saturable component operating at low concentrations and a diffusion-like one at high concentrations. Treatment of cotyledons with carbonyl-cyanide-*m*-chlorophenylhydrazone and *p*-chloromercuribenzenesulfonic acid reduced D-mannose-uptake rates by about 35% and 35–65%, respectively. No difference in the uptake rates was observed in the presence of D-galactose or 3-O-methylglucose. D-Mannose uptake was not very much affected by pH. The optimum pH for its uptake was 6.5 while at pH 8.5 its uptake was reduced by 22%. D-Mannose addition to fenugreek cotyledons did not induce alkalinization of the medium. Furthermore, low turgor, which enhances proton/sugar cotransport, decreased D-mannose uptake while the uptake of 3-O-methylglucose was increased. The rate of D-mannose uptake by fenugreek cotyledons depended on the hours of imbibition. These changes of uptake were not followed by analogous changes in the turgor pressure (ψ_p) of fenugreek cotyledons, which remained fairly constant. Results indicate that D-mannose is partially taken up by a carrier which has high specificity for D-mannose, but not by a H^+-sugar cotransport system. It is further concluded that the carrier plays an important role in switching on and off the uptake capacity of fenugreek cotyledons during seedling development.

Key words: D-Mannose transport – Seed germination – Sugar transport – Transport (sugar) – *Trigonella*

Introduction

Galactomannan is the main reserve of endospermic legumes. After germination, galactomannan is hydrolysed by the action of α-D-galactosidase (EC 3.2.1.22), endo-β-D-mannanase (EC 3.2.1.78) and exo-β-D-mannanase (EC 3.2.1.25; Reid and Meier 1973; Reid et al. 1977). The hydrolysis products are mainly D-galactose and D-mannose which are transported to the embryo where they are converted to sucrose, starch and new cell material (Reid 1971; Seiler 1977; Spyropoulos and Lambiris 1980). Spyropoulos and Reid (1988) have shown that water stress imposed on germinated fenugreek seeds reduced galactomannan hydrolysis, although the in-vitro activities of the endospermic hydrolases were not affected. They also reported that the amount of reducing sugars in the endosperm was higher under conditions of water stress and put forward the hypothesis that the in-vivo inhibition of galactomannan degradation might be attributed partly to the high galactose and mannose concentrations found in the endosperm. Furthermore, Spyropoulos (1986) has shown that stressed fenugreek embryos can take up only limited amounts of galactose even when the galactose concentration of the medium is high.

The mechanism of D-galactose uptake by fenugreek embryos has been studied by Uebelmann (1978) while that of D-mannose is completely unknown. The membrane transport of sugars has been extensively studied in several plant materials (Reinhold and Kaplan 1984). Thus, sucrose uptake

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Abbreviations and symbols: CCCP = carbonylcyanide-*m*-chlorophenylhydrazone; DTT = dithiothreitol; 3-OMG = 3-O-methylglucose; PCMBS = *p*-chloromercuribenzenesulfonic acid; ψ = water potential; ψ_s = osmotic potential; ψ_p = turgor pressure

occurs via a carrier-mediated sucrose H^+/co-transport system (Giaquinta 1983 and literature therein). Glucose is taken up by a carrier but in several plant materials is not transported via H^+/co-transport (Giaquinta 1983; Griffith et al. 1987). Ubelmann (1978) in his studies on the uptake of D-galactose by fenugreek embryos concluded that D-galactose is taken up by non-facilitated passive diffusion.

The lack of information on the mechanism of D-mannose uptake by plant tissues and the possible involvement of its uptake by the embryo in the control of endosperm galactomannan mobilisation in fenugreek prompted us to investigate D-mannose transport.

Material and methods

Plant material. Fenugreek (*Trigonella foenum-graecum* L.; the same batch as used previously; Spyropoulos and Reid 1988) seeds were germinated under sterile conditions in the dark at 25±1° C as previously described (Spyropoulos and Reid 1988).

Sugar uptake. Sugar uptake took place in a dark room under a green safelight at 25±1° C. After the required time of imbibition, ten pairs of cotyledon were gently dried with absorbant paper, weighed and equilibrated in a medium containing 100 mM mannitol, 20 mM 2-(N-morpholino)ethanesulfonic acid (Mes)-NaOH buffer and 2 mM $CaCl_2$ (pH 6.5) for 30 min. The cotyledons were then transferred to the incubation medium which contained [U-^{14}C]-D-mannose or [U-^{14}C]-3-O-methylglucose (3-OMG; 3.7–11.3 kBq per ml and 37 kBq per 3 ml, respectively). Mannitol (as osmoticum) was added in various amounts to the incubation medium so that the total sugar concentration (mannose or 3-OMG + mannitol) was 100 mM. In water-stress experiments the water potentials of the equilibration and uptake solutions were adjusted by the addition of adequate amounts of polyethyleneglycol (PEG) 3350. After 30 min incubation (D-mannose uptake was linear for 45 min) cotyledons were rinsed three times (1-min rinses) in the preincubation medium at 0° C and extracted in 1 ml H_2O_2:$HClO_4$ 3:2 (v/v) at 50° C for 4 h. An aliquot of the extract was used for radioactivity measurements using a scintillation counter and Omnifluor in Triton X100-toluene as scintillant. The activity which remained in the cotyledons after extraction was negligible. The [U-^{14}C]-D-mannose (11.3 $GB_2 \cdot mmole^{-1}$) and 3-OMG (11.9 $GB_2 \cdot mmole^{-1}$) were purchased from Amersham (Amersham, UK) and New England Nuclear (Boston, Mass., USA) respectively.

Effect of pH on D-mannose uptake. Ten cotyledon pairs which were isolated from seeds that had been imbibed for 40 h, were preicunbated in 100 mM mannitol solutions containing various buffers: 10 mM citrate-20 mM Na-phosphate (pH 4.5), 20 mM Mes-NaOH (pH 5.5, 6.5), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-NaOH (pH 7.5) and 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl (pH 8.5). After 30 min preincubation the cotyledons were incubated for 30 min in the same buffered solution containing 1 mM [U-^{14}C]-D-mannose and then processed as described above.

Proton flux. One hundred cotyledon pairs (about 1 g total fresh weight) were isolated after 40 h imbibition and preincubated for 30 min in 2 mM $CaCl_2$, 1 mM $MgCl_2$ (with or without inhibitor). The cotyledons were placed in a 10-ml vial containing 5 ml of the same medium (pH 6.3) and the solution stirred with a magnet. The pH of the medium was measured at 1- to 5-min intervals using a combination Ingold (Frankfurt, FRG) electrode until the pH of the medium was stabilised (1 h). Sugar transport was initiated by the addition of the sugar dissolved in 200 µl of the medium so that the final sugar concentration was 20 mM.

Galactomannan hydrolysis. Galactomannan hydrolysis in vivo was determined gravimetrically as described previously (Spyropoulos and Reid 1985).

Water relations. Turgor pressure (ψ_p) was estimated, according to the equation $\psi = \psi_s + \psi_p$, from the corresponding values of water potential (ψ) and osmotic potential (ψ_s) which were measured using a Wescor (Logan, UT, USA) Dew Point hygrometer. Three to five cotyledon pairs were gently dried and their water potential was measured. The same cotyledons were quickly frozen and their osmotic potential was measured upon thawing (Tomos et al. 1984). The water relations of fenugreek cotyledons were measured at the end of the preincubation period.

Results

Comparison of detached and attached cotyledons. The rates of D-mannose uptake by attached and detached cotyledons of seeds imbibed for 40 h were similar (25 and 23 $nmol \cdot (cot\ pair)^{-1} \cdot h^{-1}$, respectively). In the experiments described below detached cotyledons were used.

Uptake kinetics. Figure 1 shows the concentration dependence of D-mannose uptake and the effect of the inhibitors carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and *p*-chloromercuribenzenesulfonic acid (PCMBS). D-Mannose uptake showed a biphasic dependence on exogenous substrate, corresponding to a high- and a low-affinity system.

Transformation of the data to a Lineweaver-Burk plot further confirms the two-component system for D-mannose uptake (Fig. 1, insert). The apparent kinetic parameters (which were calculated using linear regression, $r_1 = 1.000$ and $r_2 = 0.994$) were $K_{m1} = 2.2$ mM and $V_{max1} = 5.6\ \mu mol \cdot g^{-1} \cdot h^{-1}$ and $K_{m2} = 33.3$ mM and $V_{max2} = 50\ \mu mol \cdot g^{-1} \cdot h^{-1}$ for the high- and low-affinity components, respectively.

p-Chloromercuribenzenesulfonic acid, a non-penetrating sulfhydryl inhibitor, seems to inhibit the sugar and amino-acid carrier by blocking its extracellular sulfhydryl groups and not the proton pump (Giaquinta 1976; Delrot et al. 1980; Lichtner and Spanswick 1981; Monoury et al. 1984; M'Batchi et al. 1987). Treatment with PCMBS decreased D-mannose uptake, showing

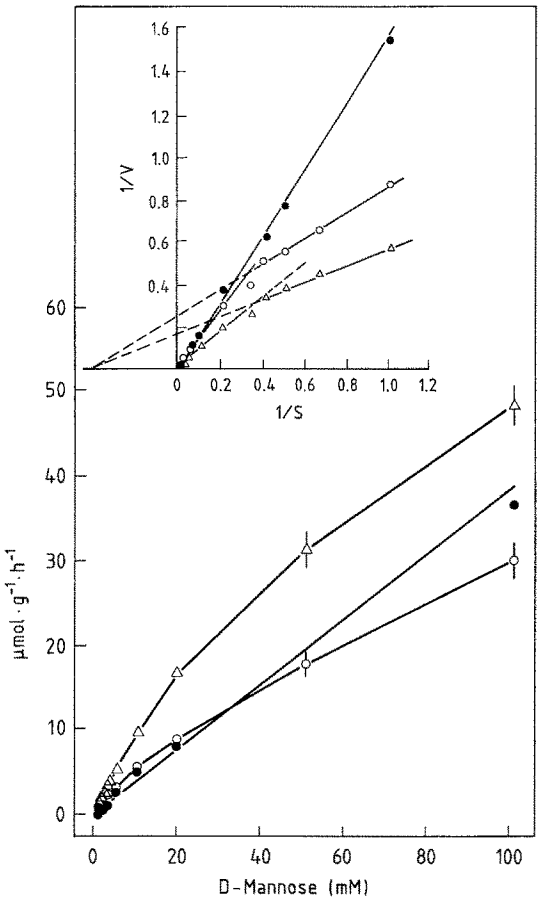

Fig. 1. Kinetics of D-mannose uptake by fenugreek cotyledons which were isolated after 40 h imbibition, under normal conditions (△–△) and in the presence of 10 µM CCCP (○–○) or 2 mM PCMBS (●–●). The CCCP was added in both the preincubation and the incubation media; PCMBS was added in the preincubation medium and after 15 min the cotyledons were washed with the preincubation medium for 15 min (three 5-min rinses) before they were transferred to the incubation medium. *Insert:* Lineweaver-Burk plot of the uptake showing control (△–△), CCCP (○–○), and PCMBS (●–●) treatments. V is expressed on a fresh-weight basis. Values are the means ±SE of four (control and CCCP treatment) or three (PCMBS treatment) experiments

diffusion kinetics (Fig. 1, insert). These results confirm that part of D-mannose uptake is carrier-mediated. However, deduction of the plot for PCMBS from the control leads to single Michaelis-Menten kinetics. Thus, the low-affinity component could be interpreted as reflecting diffusion.

Treatment with CCCP, a metabolic uncoupler which dissipates the proton gradient across the plasmalemma, reduced the uptake rate of D-mannose by about 35%, indicating that its uptake depended on metabolic energy. The action of CCCP as a penetrating sulfhydryl inhibitor (Daie and Wilusz 1987) should be excluded since the addition of dithiothreitol (DTT) to the incubation medium

Table 1. D-Mannose uptake by detached cotyledons of fenugreek as affected by the presence of D-galactose and 3-O-methylglucose. Ten cotyledon pairs which were isolated from seeds after 40 h imbibition were incubated in 2 or 5 mM [U-^{14}C]-D-mannose in the presence or absence of 2 and 10 mM galactose or 3-OMG. The values are the mean±SE from at least three experiments

D-Mannose (mM)	D-Galactose (mM)	3-OMG (mM)	D-Mannose uptake (µmol·g^{-1}·h^{-1})
2	–	–	2.76±0.10
2	2	–	3.01±0.15
2	10	–	3.03±0.17
5	–	–	6.72±0.61
5	2	–	6.21±0.20
5	10	–	5.96±0.35
2	–	2	3.10±0.42
2	–	10	3.07±0.25

of CCCP-treated cotyledons did not result in recovery of D-mannose uptake (Table 3).

D-Galactose and 3-OMG did not affect significantly the uptake of D-mannose (Table 1), indicating further a high carrier specificity and also that D-galactose is taken up by fenugreek cotyledons by a transport system different from that for D-mannose uptake.

Effect of pH. Proton availability has a marked effect on sucrose uptake (Giaquinta 1983) but not on the D-glucose uptake of some systems (Griffith et al. 1987). The pH dependence of sucrose uptake has been related to the requirement of protons as co-transport substrate (Komor 1982). D-Mannose uptake was not affected substantially by pH. The maximum rate of uptake was observed at pH 6.5 and at pH 8.5 the rate of uptake was reduced by 22% (Fig. 2). The amount of label excreted for 30 min, after loading cotyledons with 1 mM [U-^{14}C]-D-mannose for 30 min, was approximately the same at pH 6.5 and 8.5 (0.09 and 0.10 µmol·g^{-1}·h^{-1}, respectively). Thus the decrease in radioactivity which was observed at alkaline pH was not due to the effect of pH on the efflux of labeled compounds.

Proton flux. One of the criteria for sugar-proton co-transport is the rapid transient alkalinisation of the tissue incubation medium upon sugar addition. D-Mannose uptake was not followed by alkalinisation of the medium (the expected pH rise, if H^+-symport occurs at 1:1 stoichiometry with D-mannose uptake, was calculated to be 0.2 pH units), while uptake of sucrose resulted in an increase of pH by 0.03 pH units. This increase started 2 min after sucrose addition to cotyledons in a non-buf-

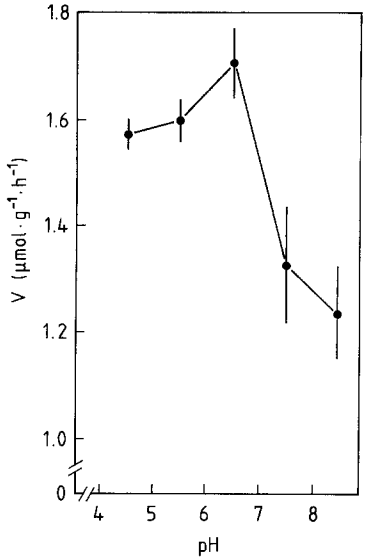

Fig. 2. Effect of pH on D-mannose uptake by fenugreek cotyledons which were isolated after 40 h imbibition from 1 mM D-mannose solution. Values are the means ± SE of four experiments

Table 2. Effect of water stress on D-mannose and 3-OMG uptake by fenugreek cotyledons. After 40 h imbibition, 10 cotyledon pairs were isolated and equilibrated for 30 min in a preincubation medium of −1.0 or −1.5 MPa using PEG 3350 as osmoticum. The uptake from 10 mM D-mannose or 3-OMG was studied as previously described. Values are the means ± SE of three experiments

ψ_{medium} (MPa)	ψ_p (MPa)	Uptake rate (nmol·(cot·pair)$^{-1}$·h^{-1})	
		D-mannose	3-OMG
−0.3	0.60	152 ± 4	28 ± 1
−1.0	0.54	136 ± 14	39 ± 6
−1.5	0.43	123 ± 8	52 ± 3

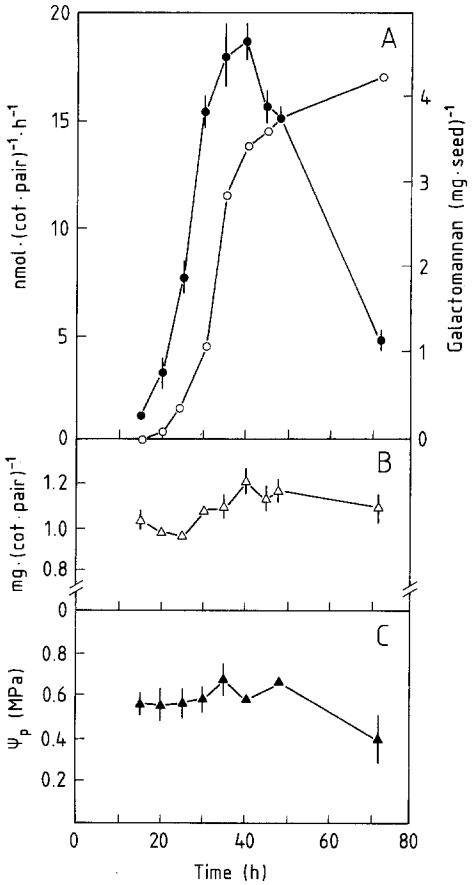

Fig. 3A–C. D-Mannose uptake (○–○) from 1 mM D-mannose solution, and galactomannan depletion (●–●, **A**), cotyledon fresh weight (**B**), and turgor pressure (**C**) as affected by the time of imbibition of fenugreek seeds. Values are the means ± SE of four (D-mannose uptake, galactomannan content and cotyledon fresh weight) or three (turgor pressure) experiments

fered solution and was dissipated after 15 min. No alkalinisation was observed upon addition of sucrose in the presence of 10 μM CCCP (data not shown).

Decrease of the cellular turgor increases the activity of the H^+-ATPase pump (Reinhold et al. 1984; Kinraide and Wyse 1986), resulting in the increase of sucrose uptake (Wyse et al. 1986). However, water stress (−1.0 and −1.5 MPa) resulted in the reduction of D-mannose uptake, while 3-OMG uptake was increased under the same stress conditions (Table 2). We used 3-OMG because it is not appreciably metabolised by plants (Cameron-Mills and Duffus 1979) and is transported by a carrier via an H^+/co-transport system (Colombo et al. 1978). These results are in accordance with the view that sucrose is transported via proton co-transport (Giaquinta 1983; Reinhold and Kaplan 1984) whereas D-mannose is not.

D-Mannose uptake as a function of the time of imbibition. Ubelmann (1978) found that D-galactose uptake by fenugreek embryos was very low before germination, increased as incubation times were extended, reaching a maximum after 40 h imbibition, and decreased thereafter. A similar pattern was followed by D-mannose uptake by fenugreek cotyledons. Up to 40 h imbibition, D-mannose uptake was parallel to the rate of galactomannan depletion (Fig. 3A). During the same period the fresh weight of cotyledons did not change substantially (Fig. 3B). Thus, the different values for D-mannose-uptake rates during seedling development should not be attributed to the sink size. Also, the changes in the turgor pressure of fenugreek cotyledons were not significant (Fig. 3C). The turgor pressure of cotyledons after 15 h imbibition

Table 3. D-Mannose uptake by fenugreek cotyledons as affected by the presence of PCMBS or CCCP. Ten cotyledon pairs were isolated from 20-, 40- and 72-h-imbibed fenugreek seeds and treated with 2 mM PCMBS as described previously. After 15 min treatment the cotyledons were washed for 3×5 min with the preincubation medium or washed for 3×1 min and then incubated in the same medium plus 20 mM DTT for 7 min and subsequently washed for 2×3 min with the preincubation medium. Then, cotyledons were transferred to the incubation medium which contained 1 mM [U-^{14}C]-D-mannose for 30 min. Ten mM CCCP was added to both the preincubation and the incubation media, while 20 mM DTT was added only in the incubation medium. The values are the mean ± SE from three experiments

Imbibition (h)	D-Mannose uptake ($\mu mol \cdot g^{-1} \cdot h^{-1}$)				
	Control	PCMBS	PCMBS + DTT	CCCP	CCCP + DTT
20	0.19 ± 0.03	0.17 ± 0.01	–	0.13 ± 0.01	–
40	1.71 ± 0.07	0.65 ± 0.06	1.78 ± 0.06	1.15 ± 0.04	0.88 ± 0.04
72	0.43 ± 0.01	0.37 ± 0.03	–	0.20 ± 0.05	–

was about 0.6 MPa and remained constant but decreased in cotyledons from seeds which were imbibed for 72 h. This turgor reduction apparently should be due to the cessation of solute import from the endosperm since after 48 h imbibition very little galactomannan was left in the endosperm (Fig. 3A). Therefore, the different uptake rates of D-mannose should not be related to the ψ_p of fenugreek cotyledons, either, but rather to some metabolic event. For example, there may be a specific transport system which is induced when galactomannan degradation starts and stops operating when galactomannan is depleted. Thus, it may be argued that the low turgor pressure of cotyledons from seeds which were imbibed for 72 h changed the properties of the plasmalemma, and consequently the function of the carrier, resulting in the reduction of D-mannose uptake. Alternatively, D-mannose uptake may be related to metabolic processes occurring in cotyledons that do not operate after a certain time.

Treatment with the non-penetrating sulfhydryl-binding inhibitor, PCMBS, decreased by 62% the rate of D-mannose uptake by cotyledons from 40-h-imbibed seeds, but it had no effect when cotyledons were isolated after 20 and 72 h imbibition (Table 3). The uptake of D-mannose completely recovered when PCMBS treatment was followed by treatment with DTT (DTT alone had no effect on D-mannose uptake). Treatment with CCCP reduced D-mannose uptake by 32–50% (Table 3). These results show that at 20 or 72 h imbibition the carrier system is not operating and D-mannose is transported by non-facilitated diffusion. This leads to the view that the carrier regulates the capacity for D-mannose uptake by fenugreek cotyledons during seedling growth. A similar situation seems to exist in *Ricinus* where the low uptake rates of sucrose by *Ricinus* cotyledons at the early and late times of imbibition was assumed to result from the net synthesis and net degradation, respectively, of the sucrose carrier (Komor 1977).

Discussion

The effect of PCMBS treatment and the uptake kinetics indicate a biphasic mechanism of D-mannose transport. The diffusion-like component may have important physiological significance where high apoplastic sugar concentrations exist. However, the importance of the carrier relative to the diffusion uptake mechanism in vivo can not be judged from our data.

Proton flux, pH studies and the turgor effect on D-mannose uptake, all point to the view that D-mannose does not use protons as the co-transport substrate. It might be argued, however, that the turgor effect is not such a strong argument against proton co-transport and that the proton symport could not be seen with mannose as easily as with sucrose if sucrose had a higher active uptake. However, the direct expenditure of energy at the plasmalemma to drive D-mannose uptake is not likely to be significant since D-mannose does not accumulate intracellularly (Reid 1971). In lucerne and carob, which also reserve galactomannan, D-mannose is immediately converted to D-mannose-6-phosphate which, in turn, is rapidly converted to fructose-6-phosphate by phosphomannoisomerase (EC 5.3.1.8), thus allowing its entry to glycolysis (McCleary and Matheson 1976). Therefore, there will be a substantial D-mannose gradient from the apoplast inward, which could provide the driving force for its uptake. The inhibition of D-mannose uptake by CCCP may be explained by its dependence on metabolic energy required for D-mannose transformations in the cell. The pH dependence of D-mannose uptake may reflect the pH dependence of the carrier.

D-Mannose accumulation in the endosperm

would decrease endosperm water potential leading to the decrease of galactomannan hydrolysis (Spyropoulos and Reid 1988). Thus, removal of D-mannose from the endosperm would ensure the hydrolysis of galactomannan. In this context, it may be argued that fenugreek cotyledons regulate galactomannan mobilisation through the regulation of D-mannose uptake.

We thank Dr. J.S. Grant Reid and Dr. C. Willmer (University of Stirling, UK) for useful discussion and Dr. K. Roubelakis (University of Crete, Greece) for the gift of [U-^{14}C]-3-O-methylglucose. A grant from the General Secretary of Research and Technology, Greece, is gratefully acknowledged.

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Received April 10; accepted May 30, 1989