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Diverse transporters for neutral amino acids in *Ricinus communis* L. seedlings

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Abstract

Uptake mechanisms for neutral amino acids were investigated by expression of mRNA isolated from seedlings of *Ricinus communis* L. in *Xenopus laevis* oocytes. After injection of mRNA from root, hypocotyl and cotyledon currents elicited by saccharose and neutral amino acids ranged from 0.3 nA up to 2 nA depending on the respective substrate and the source of mRNA. These currents were due to expression of low affinity uptake mechanisms and the K_M values found for amino acid induced charge flow range from 1 to 2 mM. The abundance and/or the specificity of the expressed mechanisms differ in the various tissues. Currents of similar magnitude were recorded for alanine and glutamine with mRNA isolated from root, hypocotyl and cotyledons. Serine and proline induced currents after injection of mRNA from hypocotyl and roots, in case of α-aminoisobutyric acid (AIB) induced currents were generally small with mRNA from all tissues tested. In addition, differential sensitivity of glutamine and AIB uptake in the high affinity range was evident towards the amino acid analogue 2-chloro-aminophenoxybutyric acid which indicated an additional set of carriers operating in the micromolar concentration range. The results suggest that multiple transporters for neutral amino acids exist in various tissues of the plant differing in specificity of charge flow and in sensitivity towards the inhibitor 2-chloro-aminophenoxybutyric acid.

Additional key words: castor bean, *Euphorbiaceae*, mRNA injection, oocyte expression, saccharose transport, tissue specificity, *Xenopus laevis*.

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Abbreviations: AIB - α-aminoisobutyric acid; K_M - Michaelis constant; PhAmBu - 2-chloro-aminophenoxybutyric acid.

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Introduction

Amino acid transport within plants is essential to ensure a balanced nitrogen supply especially for growing tissues. Indeed, nitrogen flow from source to sink via the phloem is exclusively in the form of amino acids, which are found in high concentration - ranging from 50 to 200 mM - in phloem sap (e.g. Allen and Raven 1987, Shelp 1987). Amino acids are also detected in the xylem in varying concentration depending on nitrogen supply (Pate 1980, Peoples *et al.* 1986). Specific carriers were postulated to mediate accumulation of amino acids into the phloem (Schobert and Komor 1989) and transfer of amino acids into the xylem (Schobert and Komor 1990).

Roots absorb amino acids with high affinity (Schobert and Komor 1987) and compete successfully with microorganisms for soil amino acids (Schobert *et al.* 1988). Indeed, amino acid uptake by the root is the dominant form of nitrogen acquisition of a non-mycorrhizal arctic sedge (Chapin *et al.* 1993). Also cotyledons and scutella possess active amino acid uptake mechanisms for absorption of amino acids which are mobilised in the endosperm (Robinson and Beevers 1981, Sopanen and Väisänen 1985).

Recently genes encoding amino acid carriers were identified by functional complementation of yeast amino acid transport mutants with *Arabidopsis* cDNA libraries (Frommer *et al.* 1993, Kwart *et al.* 1993, Hsu *et al.* 1993). For further understanding of the amino acid transport mechanisms on the molecular level operating in different plant tissues we aimed to express amino acid transport activities in *Xenopus* oocytes, a technique well established to analyse amino acid transporters from animal tissues (Aoshima *et al.* 1988) which is also applicable to molecular characterisation of plant ion channels and transporters with coupled net charge flow (Schroeder 1994). To this end mRNA from *Ricinus* seedlings was isolated because of the well known properties of amino acid transport in this system (Schobert and Komor 1987, 1989, 1990) and injected into oocytes. In addition an amino acid analogue with high affinity to a neutral amino acid uptake mechanism in *Egeria* (Petzold *et al.* 1991) was used to further dissect amino acid carriers from root and cotyledon. In this paper results are described which argue for differential mechanisms for uptake of neutral amino acids in roots and cotyledons in the micromolar and millimolar concentration range.

Materials and methods

Growth of seedlings: Seeds of *Ricinus communis* cv. Sanguineus were purchased from Jelitto Staudensamen, Schwarmstedt, FRG. Intact seeds were soaked overnight in an excess of water (50 seeds in 1 dm^3) which was changed 1 h after soaking. The next day fungus-free seeds were surface sterilised in 0.3 % chinosol (8-hydroxy-chinolin-sulphate) for 10 min, washed with 3 changes of sterile water, germinated at 27 °C on 2 % agar for 3 d and then transferred to aerated liquid culture with 0.5 mM

$CaCl_2$ (Schobert and Komor 1987). After another 3 d of growth seedlings were used for uptake experiments.

For preparation of mRNA seeds were germinated in wet autoclaved vermiculite saturated with water and grown at 29 °C for 4 d (mass of cotyledons approximately 100 mg).

Uptake experiments: Six-d-old seedlings were used. Roots were preincubated for 2 h in 0.5 mM $CaCl_2$ before transfer (1 root per 10 cm^3) to uptake buffer (5 mM potassium phosphate, pH 6, 0.5 mM $CaCl_2$) and incubation in a gyrotory water bath at 27 °C with gentle shaking. Uptake was started by addition of ^{14}C-labelled amino acids. Samples from the medium were withdrawn in 10 min intervals in duplicates and remaining ^{14}C quantified by scintillation counting.

Cotyledons were removed from the endosperm and collected in uptake buffer at 4 °C. 6 cotyledons were transferred to 20 cm^3 uptake buffer, incubated in a gyrotory water bath at 27 °C with gentle shaking. Uptake was started by addition of ^{14}C-labelled amino acids. 2 cotyledons each were removed after 10, 30 and 60 min, washed in ice-cold water for 30 s, blotted dry on paper towel, weighed and extracted for 2 h (1 cotyledon per 10 cm^3) in *Rotiszint 2211* (*Roth*, Karlsruhe, Germany) before scintillation counting.

mRNA isolation: Cotyledon, hypocotyl and root tissues, respectively, were frozen and ground to fine powder in liquid nitrogen. About 8 g of tissue was homogenised in 20 cm^3 guanidinium thiocyanate solution. After ultracentrifugation (100 000 *g*) on 5.7 M CsCl for 16 h, poly(A)mRNA was isolated by oligo(dT)cellulose chromatography (Maniatis *et al.* 1982). The concentration and purity of the mRNA preparation was determined photometrically at 260 and 280 nm.

Preparation of oocytes: Female adult *Xenopus laevis* were purchased from Hamamatsu Seibutu Kyozai (Hamamatsu, Japan). Ovaries were removed from anaesthetised frogs and oocytes were detached manually from the inner ovarian epithelium and follicular envelope (Kusano *et al.* 1982). The oocytes were injected with mRNA (about 50 ng per oocyte) and incubated in a modified Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 7.5 mM Tris, pH 7.6) containing 25 mg dm^{-3} penicillin, 50 mg dm^{-3} streptomycin and 1 % calf serum at 16 - 18 °C for 2 - 4 d before electrophysiological measurements.

Electrophysiology: The respective oocyte was placed on the bottom of a plastic chamber, which was continuously perfused via a gravity-feed system with choline ringer solution (115 mM choline chloride, 1 mM KCl, 1.8 mM $CaCl_2$, 5 mM Mops-Tris, pH 6.0) with or without saccharose or amino acids. In preliminary experiments frog ringer (115 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 5 mM Tris, pH 6.0) was used. For voltage clamp measurements the oocyte was impaled with 2 micro-electrodes filled with 3 mM KCl, one for monitoring membrane potential and the other for injecting current using a voltage clamp amplifier *CEZ-1100* (*Nihon Koden Kogyo*, Tokyo, Japan). The current was filtered by a P-84 filter (*NF Electronic*

Instruments, Kanagawa, Japan). For experiments the oocyte membrane potential was clamped to -50 mV.

Results

2-chloro-aminophenoxybutyric acid (PhAmBu) inhibits uptake of neutral amino acid in a tissue specific manner: Roots and cotyledons of *Ricinus* seedlings take up neutral amino acids by active transport systems with broad specificities (Schobert and Komor 1987, Robinson and Beevers 1981). Clear-cut analysis of the specificity of the involved systems is hampered by the fact that the uptake is not saturable and thus inhibition by competing neutral amino acids is low. Therefore, the amino acid analogue PhAmBu was used which inhibits uptake of AIB at micromolar concentration in *Egeria* (Petzold *et al.* 1991). To test whether the respective amino acid uptake activities in roots and cotyledons of *Ricinus* seedlings are related the effect of PhAmBu was analysed on uptake of glutamine and AIB. Equimolar concentration (0.1 mM) PhAmBu inhibited both uptake of glutamine (46 %) and AIB (86 %) by the roots (Table 1). On the other hand, at the cotyledon PhAmBu reduced uptake of glutamine (39 %) but uptake of AIB was not affected. Inhibition by PhAmBu of neutral amino acid uptake is not due to competition for energy because uptake was not impaired of the basic amino acid arginine (Tables 1, 2).

Table 1. The uptake of glutamine, AIB and arginine [pmol g^{-1}(f.m.) s^{-1}] by roots as affected by PhAmBu. ^{14}C-labelled amino acids (1 kBq 100 $nmol^{-1}$) were offered to the roots at 0.1 mM, PhAmBu was added at 0.1 mM. The uptake rates were determined from ^{14}C depletion in the medium over a 30 min period (n = 5, mean ± SD).

	Control	PhAmBu	Inhibition [%]
Glutamine	116 ± 17	63 ± 14	46
AIB	71 ± 13	10 ± 5	86
Arginine	75 ± 9	64 ± 3	14

Table 2. The uptake of glutamine, AIB and arginine [nmol g^{-1}(f.m.) s^{-1}] by cotyledons as affected by PhAmBu. Cotyledons were incubated in 0.1 mM ^{14}C-labelled amino acids (1 kBq 100 $nmol^{-1}$), PhAmBu was added at 0.1 mM. The uptake rates were determined from ^{14}C in the cotyledons harvested after 10, 30 and 60 min (n = 5, mean ± SD).

	Control	PhAmBu	Inhibition [%]
Glutamine	87 ± 11	52 ± 6	39
AIB	23 ± 1	24 ± 1	none
Arginine	56 ± 2	76 ± 9	none

Expression of neutral amino acid transport activity from *Ricinus* seedlings in *Xenopus* oocytes: Expression of *Ricinus* mRNA was envisaged in *Xenopus laevis* oocytes for

further molecular characterisation of the uptake mechanisms for neutral amino acids. Oocytes showed amino acid dependent membrane currents (Table 3) due to endogenous sodium dependent amino acid transport activities (Campa and Kilberg 1989) when incubated in conventional "frog ringer" which contains sodium ions. Much less endogenous amino acid transport activities were expected (Taylor *et al.* 1989) when sodium ions were replaced by choline. Indeed, both the total number of oocytes showing amino acid dependent membrane currents were reduced as well as the number of frogs which possess such oocytes (Table 3). Saccharose dependent currents in oocytes were reliably below 0.2 nA (Table 3), because oocytes lack endogenous saccharose transporters. Therefore, currents elicited by saccharose in injected oocytes were routinely analysed for control of expression of *Ricinus* mRNA.

Table 3. Electrical responses of oocytes incubated in sodium ions or choline. 10 mM saccharose or amino acids, 2 mM each of glutamine, glutamate, alanine, arginine and histidine, were applied to oocytes incubating either in ringer with Na^+ or choline. The number of oocytes which showed membrane currents greater than 0.2 nA is shown as well as the number of frogs from which these oocytes derived. The number of total oocytes and frogs used for analysis is given in brackets.

		Oocytes	Frogs
Na^+	saccharose	0 (14)	0 (6)
	amino acids	6 (10)	3 (4)
Choline	saccharose	0 (27)	0 (7)
	amino acids	1 (14)	1 (4)

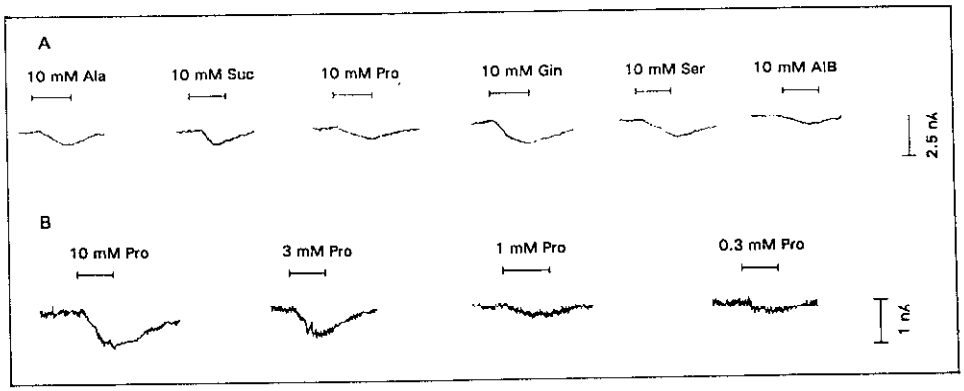

Fig. 1. Typical membrane currents elicited by amino acids and saccharose in a mRNA-injected oocyte. All traces were obtained under voltage clamp at -50 mV in choline ringer solution at pH 6. Each set of responses was obtained from an individual oocyte. The *upper bars* indicate the application period of the respective solution. Inward currents are downward. *A* - oocyte injected with cotyledon mRNA. *B* - oocyte injected with hypocotyl mRNA.

Oocytes injected with mRNA isolated from 5-d-old *Ricinus* seedling tissue express amino acid dependent membrane currents within 2 to 4 d after injection. Typical responses to saccharose and to the neutral amino acids alanine, proline, glutamine, serine and AIB are shown in Fig. 1*A* for an oocyte injected with cotyledon mRNA. With 10 mM solutions the currents ranged from about 0.5 nA in case of AIB to 1 nA in case of glutamine.

The elicited currents depend on the applied concentration. Typical recordings are shown for an oocyte challenged with proline in the concentration range from 0.3 mM to 10 mM (Fig. 1B). A steady increase of the current was evident from about 0.2 nA at 0.3 mM up to 1 nA at 10 mM. Similar results were obtained when the concentration dependence of proline, serine and glutamine were analysed in greater detail (Fig. 2). Assuming Michaelis-Menten kinetics K_M-values which were derived from Lineweaver-Burk plots of the amino acid dependent currents were found in the mM range: proline 1.2 mM, glutamine 1.5 mM and serine 2.1 mM.

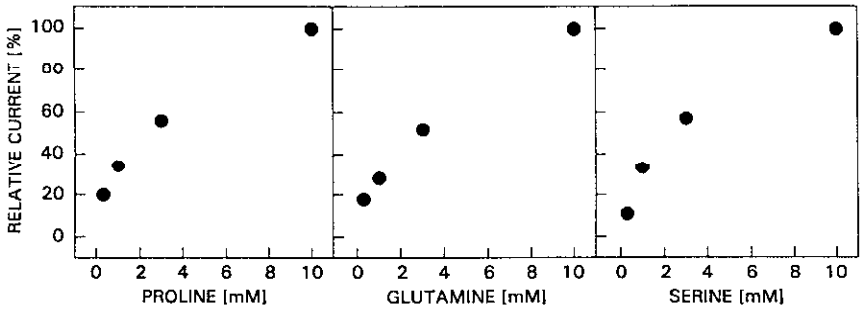

Fig. 2. Concentration dependence of membrane currents elicited in mRNA-injected oocytes by proline, glutamine and serine. Mean relative currents for the respective amino acid were obtained in concentration series from at least 4 individual oocytes. Membrane currents obtained at 10 mM were set to 100 %.

Comparison of responses elicited in oocytes injected with mRNA isolated from cotyledons, hypocotyl and roots: Irrespective of the tissue used for mRNA isolation - cotyledon, hypocotyl or root - currents were recorded for injected oocytes in response to saccharose and the neutral amino acids glutamine, proline, alanine, serine and AIB (Table 4). These currents typically ranged from 0.4 nA (AIB) to 1.9 nA (serine), but were not exclusively due to expression of plant transport systems. Though at lower level, currents were also recorded for control oocytes. Therefore, currents of injected oocytes were corrected for currents found in control oocytes to allow an analysis of the *Ricinus* mRNA-dependent currents (Fig. 3). Thus, tissue-dependent differences were found in the level of currents elicited by saccharose, proline and serine. In case of saccharose mRNA from cotyledon and root allowed currents of 0.5 nA and 0.4 nA. On the other hand, proline and serine elicited currents ranging from 0.8 nA up to 1.1 nA due to mRNA from hypocotyl and root. In contrast, the level of currents elicited by glutamine (0.8 to 1 nA), alanine (0.6 to 0.8 nA) and AIB (0.3 to 0.5 nA) were not affected by the source of mRNA.

Table 4. Electrical currents [nA] of oocytes injected with mRNA isolated from various tissues of *Ricinus* seedlings. Saccharose and amino acids were applied to the oocytes at 10 mM in choline ringer. For control water and uninjected oocytes were used from the same frog, respectively. The standard deviation is shown, the number of tested oocytes is given in brackets; the significance of differences was tested with Student's *t*-test ($P > 0.1$ not significant, $0.1 > P > 0.01$ significant, $P < 0.01$ highly significant).

		Injected	Control	*P*
Cotyledons	saccharose	0.58 ± 0.45 (8)	0.09 ± 0.13 (6)	0.025
	glutamine	1.09 ± 0.23 (11)	0.23 ± 0.28 (7)	0.0016
	proline	0.48 ± 0.56 (7)	0.24 ± 0.28 (7)	0.315
	alanine	0.79 ± 0.64 (10)	< 0.10 (5)	< 0.001
	serine	0.80 ± 0.38 (8)	0.55 ± 0.45 (5)	0.282
	AIB	0.54 ± 0.41 (7)	0.05 ± 0.09 (4)	0.046
Hypocotyls	saccharose	0.27 ± 0.33 (5)	0.15 ± 0.23 (6)	0.495
	glutamine	1.58 ± 0.43 (7)	0.62 ± 0.18 (7)	< 0.001
	proline	1.16 ± 0.82 (7)	0.48 ± 0.13 (6)	0.071
	alanine	0.94 ± 0.43 (10)	0.31 ± 0.42 (8)	0.035
	serine	1.16 ± 0.69 (7)	0.38 ± 0.28 (7)	0.017
	AIB	0.46 ± 0.31 (5)	0.14 ± 0.15 (6)	0.051
Roots	saccharose	0.50 ± 0.39 (5)	0.08 ± 0.17 (5)	0.058
	glutamine	1.43 ± 0.44 (7)	0.63 ± 0.32 (6)	0.0036
	proline	1.19 ± 0.34 (7)	0.39 ± 0.21 (7)	< 0.001
	alanine	0.82 ± 0.37 (6)	0.16 ± 0.17 (4)	0.011
	serine	1.85 ± 0.78 (8)	0.78 ± 0.48 (6)	0.012
	AIB	0.38 ± 0.31 (6)	< 0.10 (6)	< 0.001

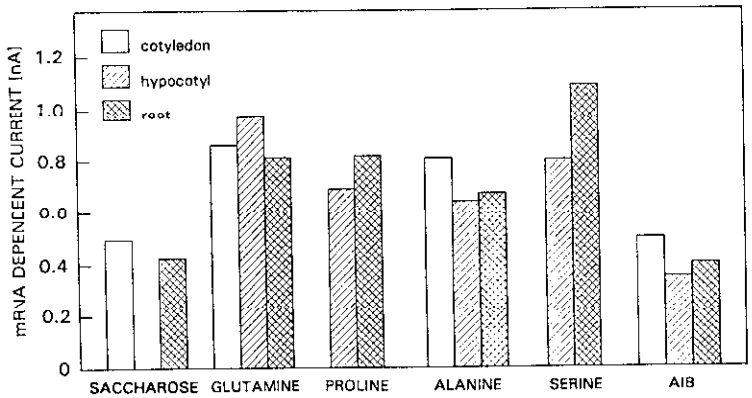

Fig. 3. Comparison of membrane currents due to injection of mRNA isolated from cotyledon, hypocotyl and root. Mean values from Table 4 were used to calculate mRNA-dependent currents by subtracting membrane currents elicited in control oocytes from membrane currents elicited in mRNA-injected oocytes which were significantly different ($P < 0.1$).

Discussion

Recordings of *Ricinus* mRNA-dependent currents were possible after application of neutral amino acids in the millimolar range. Using this approach evidence was obtained for diverse sets of neutral amino acid transporters expressed in cotyledon, hypocotyl and root tissue. Serine and proline elicited only low currents in oocytes injected with cotyledon mRNA. Consistently higher currents were recorded for oocytes injected with hypocotyl and root mRNA. On the other hand, current levels due to application of glutamine, alanine and AIB were not affected by the source of mRNA. This finding may be explained either (*1*) by the low abundance in cotyledons of a mRNA encoding a transporter with preference for serine and proline or (*2*) by differential specificities of the neutral amino acid transporter expressed in root and hypocotyl.

The amino acid analogue PhAmBu which inhibits strongly uptake of neutral amino acids in *Egeria densa* (Petzold *et al.* 1991) also affected uptake of glutamine and AIB by roots and cotyledons of *Ricinus* seedlings. Whereas the inhibitory effect on glutamine uptake was comparable both in roots and cotyledons, AIB uptake into the roots was strongly inhibited but not uptake into the cotyledons

This observation suggests that in the μM concentration range where PhAmBu inhibition was tested differences exit in the specificities and/or mechanisms of neutral amino acid transporters operating in the roots and cotyledons with respect to glutamine and AIB. These differences were not noted in oocytes injected with mRNA and challenged with 10 mM glutamine and AIB. This may indicate different mechanisms operate in the μM and the mM concentration range. Unfortunately it was not possible to test the effect of PhAmBu on currents elicited by neutral amino acids in oocytes injected with *Ricinus* mRNA, because this analogue is only soluble at concentration below 1 mM. Indeed, a biphasic concentration dependence was described for amino acid uptake by roots in *Ricinus* (Schobert and Komor 1987), namely Michaelis-Menten type kinetics below 0.5 mM and a non-saturable component up to 50 mM. This phenomena was discussed to be due either to multiple or multiphasic uptake mechanisms (Nissen 1987). The above findings as well as results on amino acid transfer through the root into the xylem (Schobert and Komor 1990) suggest multiple mechanisms that cause the observed complex kinetics. High affinity amino acid uptake mechanisms in parenchyma and rhizodermal cells may retrieve amino acids from the apoplast or absorb amino acids from the soil available only at low concentration (Schobert and Komor 1987). Low affinity mechanisms may be engaged in phloem loading as postulated by Van Bel (1986).

The ability to express cRNA encoding hexose carriers from *Arabidopsis* (Boorer *et al.* 1992) and *Chlorella* (Aoshima *et al.* 1993) suggests expression in oocytes may be used to clone plant transport systems. When compared to currents elicited by amino acids in oocytes injected with mRNA of animal origin which may well be above 10 nA (Aoshima *et al.* 1992) the currents around 1 nA which were elicited due to expression of plant mRNA are low. Nevertheless, detection of increased uptake of radiolabelled amino acids into oocytes injected with a cRNA library which was successfully used to clone a Na^+-independent neutral amino acid transporter from rat

kidney (Tate *et al.* 1992) may also work to identify plant genes encoding amino acid transporters. Thus, plant amino acid transporters may be cloned using specific amino acids which so far have not been found via complementation of yeast mutants (Frommer *et al.* 1993, Hsu *et al.* 1993, Kwart *et al.* 1993).

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