Biochimica et Biophysica Acta, 984 (1989) 57–62
Elsevier

BBAMEM 74526

Mobilisation of vacuolar amino acids in leaf cells as affected by ATP and the level of cytosolic amino acids: ATP regulates but appears not to energize vacuolar amino-acid release

Karl-Josef Dietz, Enrico Martinoia and Ulrich Heber

Institute of Botany and Pharmaceutical Biology of the University, Würzburg (F.R.G.)

(Received 9 March 1989)

Key words: Amino acid transport; ATP stimulation; Mesophyll cell; Vacuole; Tonoplast; (Barley); (*H. vulgare*)

The vacuoles of mesophyll cells are capable of storing amino acids which are mobilized on demand. An amino-acid carrier was characterized by studying the efflux of vacuolar amino acids from isolated mesophyll vacuoles using the silicon oil layer centrifugation technique. Efflux was slow in the absence of ATP. It was stimulated by a factor of more than 5 by the addition of ATP. A similar stimulation was brought about by adenylyl imidodiphosphate (Ado*PP*[NH]*P*), suggesting that efflux activation by ATP did not involve ATP hydrolysis. GTP, CTP, UTP and ADP were not effective, or were much less effective than ATP, in activating the amino-acid transport system. Neutral amino acids inhibited ATP-stimulated amino-acid efflux by an allosteric mechanism. External leucine, valine, phenylalanine and methionine were more effective in inhibiting efflux than alanine, arginine, lysine or glutamic acid when present outside the vacuole. Protein-modifying agents such as *N*-ethylmaleimide and *p*-chloromercuriphenylsulfonic acid activated amino-acid efflux from the vacuoles, whereas proteinase treatment led to an inhibition. Stimulation by ATP of amino-acid export from the vacuole and inhibition by specific amino acids may be part of a mechanism to stabilize cytoplasmic amino-acid levels during protein synthesis.

Introduction

In well-aerated soils, nitrogen is taken up by the roots of plants mainly as nitrate. In plants such as barley, it is transported to the leaves and either stored in the vacuoles of mesophyll cells [1] or reduced in their cytosol to nitrite, which is further reduced in the chloroplasts to ammonia. The latter is incorporated into keto acids to form amino acids from which proteins are assembled. Average amino-acid concentrations in leaves of barley or spinach grown under optimum conditions may range from 40 to 60 mmol $\cdot l^{-1}$. When protein synthesis is limited by the availability of nutrients such as S, concentrations of amino acids increase to levels as high as 150 mmol $\cdot l^{-1}$ [2]. After mineral deficiency is relieved, amino acids are released from the vacuoles and utilized in the cytoplasm for protein synthesis [3]. To be stored in the vacuoles or to be released, they must cross the biomembrane barrier of the tonoplast. For yeast vacuoles, specific carrier systems have been described in the tonoplast which catalyze transport of basic amino acids [4,5]. Information is also available for amino-acid transport into the vacuoles of eucaryotic microorganisms [6]. However, little is known about amino-acid transport into the vacuoles of leaf cells and no information is at hand on how amino acids stored in the vacuoles are mobilized on demand.

Material and Methods

Plant growth

Barley (*Hordeum vulgare*, var. Gerbel) was grown in a growth chamber; the light regime was 14 h light and 10 h dark, and the temperature was 18°C in the dark and 20°C in the light. Primary leaves of 10-day-old plants were harvested at the beginning of the light period.

Isolation of vacuoles

Protoplasts and vacuoles were isolated as described by Martinoia et al. [1] and by Kaiser et al. [7]. Vacuoles

Abbreviations: Ado*PP*[NH]*P*, adenylyl imidodiphosphate; NEM, *N*-ethylmaleimide; pCMBS, *para*-chloromercuriphenylsulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: K.-J. Dietz, Institut für Botanik und Pharmazeutische Biologie der Universität, Mittlerer Dallenbergweg 64, 8700 Würzburg F.R.G.

were liberated from the protoplasts by mechanical lysis. For this, the protoplasts were suspended in a solution of 400 mmol · l^{-1} sorbitol/30 mmol · l^{-1} Hepes/KOH (pH 7.8)/1 mmol · l^{-1} $MgCl_2$/1 mmol · l^{-1} $CaCl_2$/2 mmol · l^{-1} EDTA/30 mmol · l^{-1} KCl/0.2% (w/v) bovine serum albumin/20% Percoll. The suspension was forced through a needle (100 × 0.9 mm), which caused disruption of the plasmalemma. Liberated vacuoles were recovered by flotation. For this, 10 ml of lysate were first overlayered with 8 ml of Percoll-free medium (as above) and then with 2 ml of glycine betaine medium (medium as above: glycine betaine substituted for sorbitol). After centrifugation for 3 min at 100 × g and 10 min at 1200 × g, vacuoles were recovered at the interphase between sorbitol medium and glycine betaine medium. Mechanical lysis was repeated until all protoplasts had been ruptured. Vacuoles were pooled, concentrated by flotation into a solution and used for the experiments.

Efflux experiments

Efflux of amino acids was measured at 20 °C by a silicon oil centrifugation technique [8,9]. For each condition and time point, five polypropylene microcentrifugation tubes (400 µl capacity) were prepared as follows: 40 µl of vacuole suspension were added to 60 µl of medium (350 mmol · l^{-1} sorbitol/45 mmol · l^{-1} potassium gluconate/30 mmol · l^{-1} Hepes-KOH (pH 7.5)/3.3 mmol · l^{-1} dithiothreitol/0.3% (w/v) purified bovine serum albumin and other solutes as indicated above, in 67% Percoll). The samples were overlayered with 150 µl phenylmethyl silicone oil (AR 200, Wacker Chemie, München, F.R.G.) and with 40 µl of H_2O on top of that. Efflux was terminated after different incubation times by centrifugation at 10000 × g for 20 s. Intact vacuoles floated through the silicone layer into the aqueous phase, which was recovered and used for the determination of amino-acid concentrations and α-mannosidase activity. When ATP was added to the bottom layer, 10 mmol · l^{-1} magnesium gluconate was also included.

Amino-acid determination

80 µl taken from the combined upper aqueous layers were added to 20 µl of 12.5% (w/v) 5-sulfosalicylic acid dihydrate, incubated at 0 °C for 30 min and centrifuged (10000 × g, 10 min). The supernatant was diluted with buffer containing 9.4 g trilithium citrate 4-hydrate/7.4 g citric acid monohydrate/0.5% (v/v) 2,2′-thiodiethanol in 1 litre.

Amino-acid concentrations were determined with an amino-acid analyzer (Biotronik, LC 5001, Maintal, F.R.G.). The method was based on liquid ion-exchange chromatography followed by detection with ninhydrin.

α-Mannosidase determinations

Two aliquots of 20 µl each were used to measure the activity of α-mannosidase. α-Mannosidase is exclusively located in the vacuole of barley mesophyll protoplasts and was used to quantify the recovery of vacuoles during silicon oil centrifugation [1,8].

Results

Vacuolar amino-acid composition

Vacuoles were isolated from barley mesophyll protoplasts and analyzed for amino-acid composition. Vacuoles which are isolated by mechanical lysis of protoplasts reveal very low contaminations with other cellular material. The activity of chloroplastic and cytosolic marker enzymes is below 1% (cf. Refs. 10 and 11). The total concentration of amino acids was 50 ± 14 mmol · l^{-1}. Fig. 1A shows that under our growth conditions, alanine was the dominant amino acid, followed by leucine, glutamine and phenylalanine. Concentrations of all other amino acids were below 3 mmol · l^{-1}. In the absence of ATP in the medium, amino acids were only slowly lost from the vacuoles. Thus, 98% of the amino acids were recovered in the vacuoles after 20 min of incubation at 20 °C. Only efflux of asparagine (16% of the initial concentration lost after 20 min of incubation) and arginine (11%) was considerable under these conditions.

ATP-induced stimulation of amino-acid efflux

However, after addition of 10 mmol · l^{-1} ATP, efflux of all amino acids was drastically stimulated (Fig. 1). During incubation, some vacuoles break and endogenous acid phosphatases are released into the medium. Therefore, a high concentration of ATP was chosen to prevent acid phosphatases from completely hydrolyzing the added ATP. Efflux was somewhat different in different preparations of vacuoles. The extremes were 45% and 80% (with an average of 60%) of the amino acids retained in the vacuoles after 20 min incubation in the presence of ATP. The variability of amino-acid efflux was not correlated with the initial amino-acid concentration in the vacuoles. This is illustrated by an example. In two extremely different preparations of vacuoles, the initial amino-acid concentrations were 80.4 and 39.9 mmol · l^{-1}. After a 20 min period of incubation in the presence of ATP, the concentrations had dropped to 39.4 and 20.1 mmol · l^{-1}. Although the absolute rates of amino-acid efflux were different in both experiments (330 and 160 nmol (10^7 vacuoles · min^{-1}), the percentage of amino acids lost was comparable (51 and 50% of the initial concentration). This was also seen when specific amino acids were compared. To compare the affinity of the transport system for specific amino acids, the initial amino-acid contents were standardized to 100%. Groups of amino acids could be distinguished which exhibited different efflux (Fig. 1B). Asparagine was released at the highest rate from the vacuole. The basic amino acids lysine, arginine

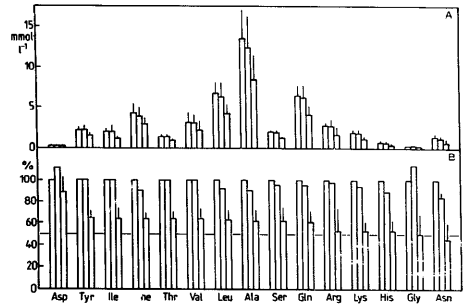

Fig. 1. Amino-acid concentrations in vacuoles isolated from barley mesophyll protoplasts (A). Vacuoles were incubated in the absence or presence of ATP and floated by silicon oil centrifugation after 2 or 20 min incubation time. The first column of each group shows the amino-acid concentration in vacuoles incubated for 2 min in the absence of ATP ('initial concentration'), the second column gives the concentration after 20 min in the absence of ATP and the third column gives the value after 20 min in the presence of 10 mmol·l^{-1} ATP. In (B), the concentrations of amino acids after 20 min without (second column) or with ATP (third column) in the medium are shown as percent of the initial amino-acid concentration (i.e., at 2 min in the absence of ATP, which was taken as 100%). This allows comparison of the efflux of the various amino acids, although their initial concentrations were different (see text). The data are those from (A).

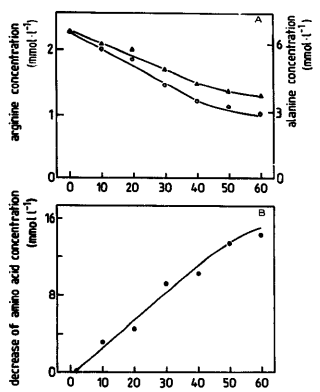

Fig. 2. Time-dependent change of vacuolar amino-acid concentrations in the presence of ATP (10 mmol·l^{-1}). In A, the change of arginine (Δ) and alanine (○) concentration is shown in dependence of incubation time. B shows the time-dependent decrease in total vacuolar amino-acid concentration. The points were calculated from the changes measured for the 12 dominant vacuolar amino acids. In this particular experiment, the rate of efflux was 45 nmol amino acids/10^7 vacuoles per min.

and histidine behaved in a similar manner. Only 50–53% were retained in the vacuoles after 20 min. With the exception of glycine, all amino acids with nonpolar side-chains exhibited comparable efflux rates. In the average, 35–40% of their content was lost from the vacuoles within an incubation period of 20 min. Aspartic acid was only slowly released. Low efflux of an acidic amino acid such as aspartic acid may be explained by the transtonoplast membrane potential. It is reported to be in the range of 10–30 mV, inside the vacuole positive [12]. At the vacuolar pH of barley mesophyll cells (pH 5.5), the side-chain of aspartic acid is negatively charged (pK = 3.86). This decreases efflux from the vacuole. The vacuolar concentration of glutamic acid was too low for efflux analysis. Fig. 2 shows the kinetics of amino-acid efflux of one particular experiment. Efflux was linear between 2 and 40 min of incubation. In the following experiments, incubation times of 2 and 20 min are routinely used, because amino-acid efflux was sufficient (usually even larger than in this experiment) and recovery of intact vacuoles was still about 50%.

Inhibition of ATP-induced efflux of amino acids by external amino acids

The ATP-induced efflux of amino acids is inhibited by the addition of leucine to the medium. Fig. 3A shows the effect of leucine on the efflux of alanine and isoleucine. Very low concentrations of external leucine were ineffective in inhibiting efflux. For 50% inhibition about 2 mmol·l^{-1} leucine were required. In the pres-

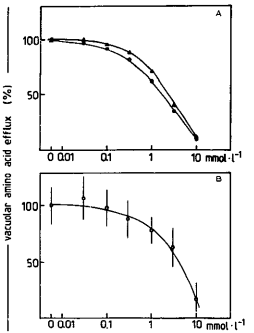

Fig. 3. Inhibition of ATP-stimulated amino-acid efflux by leucine. Leucine was added to the medium at concentrations of 0.03, 0.1, 0.3, 1, 3 and 10 mmol·l^{-1}. In A, efflux of alanine (Δ) and isoleucine (○) was studied as a function of the leucine concentration in the medium. In B, the mean efflux of 12 amino acids (Ala, Arg, Asn, Gln, His, Ile, Lys, Phe, Ser, Thr, Tyr, Val) is shown as a function of the leucine concentration in the medium.

ence of 10 mmol·l^{-1} leucine, efflux of alanine and isoleucine was reduced by more than 90%. It is important to notice that the intravacuolar concentration of leucine in these experiments was above 5 mmol·l^{-1}. Inhibition of efflux therefore cannot be explained on the basis of competition for binding sites of an unspecific amino acid transporter. As shown in Fig. 3B, the inhibitory action of leucine on amino-acid efflux is not limited to alanine and isoleucine. Although alanine is the dominant vacuolar amino acid (Fig. 1), the concentrations of arginine, glutamine, phenylalanine and valine are also above 2.0 mmol·l^{-1}. The inhibitory effect of external leucine on the averaged efflux of 12 amino acids is not very different from the efflux of alanine and isoleucine (Fig. 3B). Leucine is not the only amino acid which can inhibit the release of vacuolar amino acids when present outside the vacuoles. Fig. 4 compares inhibition of efflux of vacuolar amino acids by different external amino acids. Nonpolar amino acids were most effective in decreasing efflux; leucine, valine and phenylalanine decreased the efflux more than methionine, isoleucine or alanine. Acidic or basic amino acids decreased the efflux to a much lesser extent than nonpolar amino acids.

Nucleotide specificity of efflux stimulation

Table I summarizes the effects of various nucleotides and of the ATP analog Ado*PP*[NH]*P* on amino-acid efflux of isolated vacuoles. ADP and GTP had no, CTP

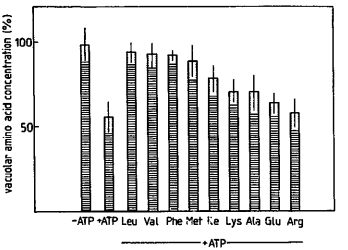

Fig. 4. Effect of different amino acids on ATP-stimulated amino-acid efflux from the vacuoles as measured after 20 min of incubation. The concentration of external amino acids was 10 mmol·l^{-1}. The first column shows internal amino-acid concentration in the absence of ATP (set to 100%), the second column in the presence of 10 mmol·l^{-1} ATP. In the latter case, the concentration is decreased owing to efflux. In the presence of external amino acids, ATP-stimulated efflux was between these values.

and UTP a small, stimulatory effect on amino-acid efflux. However, Ado*PP*[NH]*P* was almost as active as ATP in stimulating amino-acid efflux. The imido group between the β- and γ-phosphate residue prevents Ado*PP*[NH]*P* from being hydrolyzed by ATPases. The stimulation by Ado*PP*[NH]*P* of amino-acid efflux indicates that binding of adenosine triphosphate is sufficient for activating amino-acid transport, and that hydrolysis of ATP is not a necessary condition. ATP appears to regulate rather than energize amino-acid transport.

Sensitivity of ATP-stimulated amino-acid efflux to protein modifiers and proteinases

The results indicate the existance of a transport system in tonoplast membranes of barley mesophyll

TABLE I

Nucleotide specificity of stimulation of amino-acid efflux

Vacuoles were suspended in a medium containing 10 mmol·l^{-1} of GTP, CTP, UTP, ADP or Ado*PP*[NH]*P*. Amino-acid content of the vacuoles was determined after 2 and 20 min of incubation at 20 °C. Content after 20 min of incubation is expressed as percent of content after 2 min of incubation. Efflux of amino acids under these conditions is compared to efflux in the presence or absence of ATP.

Efflux condition	% amino acids retained in the vacuoles
Without ATP	98 ± 10
ATP	59 ± 7
GTP	93 ± 4
CTP	87 ± 5
UTP	87 ± 3
ADP	93 ± 4
Ado*PP*[NH]*P*	72 ± 6

TABLE II

Effects of p-chloromercuriphenylsulfonic acid, N-ethylmaleimide and of proteinases on amino-acid efflux of isolated vacuoles

Vacuoles were incubated for 2 and 20 min at 20 °C. Content after 20 min of incubation is expressed as percent of content after 2 min of incubation. Proteinases were added at a concentration of 0.5 mg·ml^{-1} in the presence of 10 mmol·l^{-1} ATP. In the case of thermolysin, 1 mmol·l^{-1} Caicium lactate was included into the medium. Amino-acid efflux after protease treatment was calculated under omission of the values for lysine and arginine (see text). DTT and BSA were omitted from the incubation medium.

Efflux condition	% amino acids retained in the vacuoles
Without ATP	97 ± 7
ATP	58 ± 5
ATP + NEM	43 ± 10
ATP + pCMBS	53 ± 11
NEM	74 ± 12
pCMBS	64 ± 14
Without ATP	97 ± 5
ATP	77 ± 7
Papain + ATP	94 ± 7
Thermolysin + ATP	96 ± 10
Trypsin + ATP	99 ± 7

cells which catalyzes export of amino acids from the vacuole and is regulated by ATP and neutral amino acids. pCMBS and NEM modify thiol groups of proteins. Whereas NEM irreversibly alkylates SH-groups, binding of pCMBS is reversible. Table II shows that both reagents increased amino-acid efflux not only in the presence, but also in the absence of ATP. In the latter case, stimulation by pCMBS of amino-acid efflux was somewhat more pronounced than stimulation by NEM. Thus, SH group modifiers could enhance amino-acid efflux and thereby mimic the effect of ATP. This result suggests that SH-groups are involved in regulating amino-acid efflux from barley mesophyll vacuoles.

The effect of three proteinases with different substrate specificity on amino-acid efflux was determined to further characterize the amino-acid carrier system. Thermolysin hydrolyzes peptide bonds involving amino groups of hydrophobic amino-acid residues such as leucine; trypsin hydrolyzes peptide bonds with carbonyl groups from arginine or lysine; papain cleaves peptide bonds which involve amino acids such as arginine, lysine, glutamic acid, histidine and tyrosine. All three proteinases reduced ATP-stimulated amino-acid efflux. Efflux in the presence of trypsin, thermolysin and papain was reduced to the rate without ATP. It is most unlikely that the liberation of amino acids by proteinases caused the decrease of efflux. The protein content of the vacuoles is 0.4 mg per 10^7 vacuoles [10]. Complete hydrolysis of all vacuolar proteins would lead to an amino-acid concentration in the vacuole suspension of less than 1 mmol·l^{-1}. However, more than 2 mmol·l^{-1} amino acids are required to reduce amino-acid efflux from vacuoles by 50%. Interestingly, efflux of arginine was not inhibited by proteinase action as was efflux of the other amino acids, but was stimulated particularly by thermolysin (40% retained after 20 min) and trypsin (41%). To a lesser extent, similar observations were made for lysine.

Discussion

Vacuoles of higher plants serve as storage compartment for ions, carbohydrates, organic acids and amino acids. All these compounds are not only deposited but also mobilized on demand. The dual role of vacuoles as sink and source compartment of plant cells requires regulation of transport across the tonoplast. The results presented in this communication characterize one such transporter and its regulation. An increase in cytosolic ATP concentration may activate the transport system, whereas an increase in cytosolic concentrations of neutral amino acids inhibits the transporter even when sufficient ATP is available for activation. The affinity of the transporter for specific amino acids was different from the specificity of inhibition by external amino acids. Alanine arginine and lysine were little effective in inhibiting amino-acid efflux, whereas leucine, phenylalanine and valine had a strong inhibitory activity. However, there was only a minor difference in relative efflux of these amino acids. Both processes had a different dependence on substrate concentration: leucine was transported with comparable efficiency when the vacuolar leucine concentration varied between 3 and 11 mmol·l^{-1}. A concentration change of the same magnitude in the medium caused a large inhibition of the transport. These observations indicate that cytosolic amino acids regulate the efflux of vacuolar amino acids by an allosteric mechanism. Efflux of amino acids was also stimulated by alkylation or oxidation (data not shown) of thiol residues, suggesting that the transporter protein may exist in conformational states which have very different activities to transport amino acids.

When plant growth is not limited by nitrogen availability and when the carbon dioxide supply is sufficient, mesophyll cells reduce carbon dioxide and nitrate in the light, and amino acids are synthesized. Chloroplast [13] and cytosolic amino-acid concentrations are high under these conditions, and efflux from the vacuole may be inhibited. When nitrogen becomes limiting, cytosolic amino acids are consumed in protein synthesis. In the presence of sufficient ATP, this decrease activates the amino acid transporter, and vacuolar amino acids can be mobilized. From work by Heber and Santarius [14], Stitt et al. [15] and Gardeström and Wigge [16] it is known that cytosolic ATP to ADP ratios are high in photosynthetic cells. Usually, the ratio is between 4 and

9, depending on conditions. ATP concentrations may be not far below 2 mmol · l^{-1}. This is likely to activate the carrier when cytosolic amino-acid concentrations are low. The dependence of amino-acid efflux on ATP concentrations could not be determined in this investigation, because some isolated vacuoles rupture during incubation and acid phosphatases are liberated into the surrounding medium. Freed phosphatases hydrolyze the ATP added to the medium. A reliable dependence of amino-acid eflux on ATP concentration has therefore not yet been obtained. In preliminary experiments, amino-acid uptake has been studied using isolated vacuoles from barley mesophyll cells. As amino acid release, uptake of [^{14}C]alanine and [^{14}C]leucine was also stimulated by ATP in the absence of Mg^{2+} ions and by Ado*PP*[NH]*P*. Addition of protonophores or ATPase inhibitors such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or NO_3^- had little or no effect on ATP-stimulated amino-acid uptake (results not shown). These results suggest that indeed activation of amino-acid transport across the tonoplast does not require ATP hydrolysis. Recently, ATP has been described by Katsuhara and Tazawa [17] to be an effector of Ca^{2+}-dependent salt tolerance in the green alga *Nitellopsis*. However in their studies, ATP and Ado*PP*[NH]*P* reduced the permeability to Na^+ of the plasmamembrane, whereas, in our experiments, ATP increased the carrier-mediated membrane permeability of the tonoplast to amino acids.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and was performed within the framework of the Sonderforschungsbereich 176 of the Bayerische Julius-Maximilians-Universität Würzburg. Excellent technical assistance by Mrs. Claudia Zink is gratefully acknowledged.

References

1 Martinoia, E., Heck, U. and Wiemken, A. (1981) Nature 289, 292–294.
2 Dietz, K.-J. (1989) J. Plant Physiol., in the press.
3 Dietz, K.-J. (1989) J. Plant Physiol., in the press.
4 Boller, W., Dürr, M. and Wiemken, A. (1975) Eur. J. Biochem. 54, 81–91.
5 Anraku, Y. (1986) in Plant Vacuoles (Marin, B., ed.), pp. 255–265, Plenum Press, Oxford.
6 Harah, J. (1986) Biochim. Biophys. Acta 864, 223–256.
7 Kaiser, G., Martinoia, E. and Wiemken, A. (1982) Z. Naturforsch. 107, 103–113.
8 Kaiser, G. and Heber, U. (1984) Planta 161, 562–568.
9 Martinoia, E., Flügge, U.-I., Kaiser, G., Heber, U. and Heldt, H.W. (1985) Biochim. Biophys. Acta 806, 311–319.
10 Kaiser, G., Martinoia, E., Schmitt, J.M., Hincha, D.K. and Heber, U. (1986) Planta 169, 345–355.
11 Dietz, K.-J., Kaiser, G. and Martinoia, E. (1988) Planta 176, 362–367.
12 Kaiser, W., Kaiser, G., Martinoia, E. and Heber, U. (1988) in The Roots in Modern Biochemistry (Kleinkauf, Von Döhren, Jaenike, eds.), pp. 721–733, Walter de Gruyter & Co., Berlin.
13 Aach, H.G. and Heber, U. (1967) Z. Pflanzenphysiol. 57, 317–328.
14 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25, 718–728.
15 Stitt, M., Lilley, R. McC. and Heldt, H.W. (1982) Plant Physiol. 70, 971–977.
16 Gardeström, P. and Wigge, B. (198) Plant Physiol. 88, 69–76.
17 Katsuhara, M. and Tazawa, M. (1987) Protoplasma 138, 190–192.