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Carrier-Mediated Uptake of 1-(Malonylamino)cyclopropane-1-Carboxylic Acid in Vacuoles Isolated from *Catharanthus roseus* Cells[1]

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ABSTRACT

The uptake of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC), the conjugated form of the ethylene precursor, into vacuoles isolated from *Catharanthus roseus* cells has been studied by silicone layer floatation filtering. The transport across the tonoplast of MACC is stimulated fourfold by 5 millimolar MgATP, has a K_m of about 2 millimolar, an optimum pH around 7, and an optimum temperature at 30°C. Several effectors known to inhibit ATPase (*N,N'*-dicyclohexylcarbodiimide) and to collapse the transtonoplastic H^+ electrochemical gradient (carbonylcyanide *m*-chlorophenylhydrazone, gramicidin, and benzylamine) all reduced MACC uptake. Abolishing the membrane potential with SCN^- and valinomycin also greatly inhibited MACC transport. Our data demonstrate that MACC accumulates in the vacuole against a concentration gradient by means of a proton motive force generated by a tonoplastic ATPase. The involvement of a protein carrier is suggested by the strong inhibition of uptake by compounds known to block SH—, OH—, and NH_2— groups. MACC uptake is antagonized competitively by malonyl-D-tryptophan, indicating that the carrier also accepts malonyl-D-amino acids. Neither the moities of these compounds taken separately [1-aminocyclopropane-1-carboxylic acid, malonate, D-tryptophan or D-phenylalanine] nor malate act as inhibitors of MACC transport. The absence of inhibition of malate uptake by MACC suggests that MACC and malate are taken up by two different carriers. We propose that the carrier identified here plays an important physiological role in withdrawing from the cytosol MACC and malonyl-D-amino acids generated under stress conditions.

The precursor of the plant hormone ethylene, ACC,[2] can be converted into a malonylated derivative (MACC) (1, 11). From our recent work (6) on the kinetics of MACC formation from ACC in protoplasts we concluded that malonylation of ACC occurs in the cytosol. Cytosolic MACC is subsequently either transported into the vacuole where it is sequestrated, or released into the apoplasmic space through the plasma membrane. Subcellular fractionations of plant cells have shown that MACC is either entirely (5), or predominantly (31), localized in the vacuole, where its concentration is much higher than in the cytosol. Under normal conditions, MACC cannot be metabolized and is sequestrated in the vacuolar compartment (6, 23). However, in some tissues fed with high levels of exogenous MACC (13), or in senescent tissues (20), the possibility exists of a conversion back to ACC through cytosolic MACC-inducible acylases. The transport of MACC into the vacuole may therefore play a central role in withdrawing MACC from the cytosol where it could otherwise be reconverted to ACC. The biochemical basis of this transport accross the tonoplast and of the vacuolar sequestration of MACC remain unknown.

In the few last years, there have been increasing numbers of publications describing transport of metabolites across the tonoplast. These include uptake of various sugars (30), amino acids (3, 12), organic acids (7, 17), phenolic compounds (21, 32) and alkaloids (8). Tophof *et al.* (31) recently mentioned the existence of energy-dependent MACC uptake by barley and wheat vacuoles. However no information is available on the biochemical characteristics of such transport. In the present paper we investigate in details the biochemical mechanisms involved in the movement of MACC across the tonoplast of isolated vacuoles. Evidence is presented for an MgATP-dependent transport mediated by a protein carrier specific for MACC and malonyl-D-amino acids.

MATERIAL AND METHODS

Cell Cultures and Isolation of Protoplasts and Vacuoles

Cell suspension cultures of *Catharanthus roseus* (L.) were grown as previously described (16).

Protoplasts were isolated from 5- to 6-d-old cultures following the procedure of Marigo *et al.* (17). For the cell wall digestion, 14 g of cells were first rinsed three times with 20 mL of 550 mM sorbitol adjusted to pH 5.5, and then incubated for 35 min at 36°C in the same solution containing 2% Caylase 345 and 1% pectolyase Y23. Protoplasts were filtered through 60 μm nylon cloth and washed three times with the sorbitol

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; dansyl-chloride, 5-(dimethylamino)naphthalene-1-sulphonylchloride; Nycodenz, 5-(*N*-2,3-dihydroxypropylacetoamido)-2,4,6-triiodo-*N,N'*-bis(2,3-dihydroxypropyl)isophtalamide.

solution after sedimenting by centrifugation at 200*g* for 1.5 min.

Vacuole isolation was performed by osmotic lysis of the plasma membrane and purification by floatation as described by Renaudin *et al.* (27). Protoplasts (10^7) were incubated for 30 min at 4°C in 2.35 mL of medium containing 200 mM sorbitol, 5 mM EDTA, and 10 mM Hepes-KOH (pH 7.0). The suspension now containing isolated vacuoles was supplemented with 8 mL of new medium in order to reach a final concentration of 350 mM sorbitol, 8.5% Nycodenz, 5 mM EDTA, and 10 mM Hepes/KOH buffer (pH 7.0). After gentle agitation, 1 mL of 550 mM sorbitol was deposited on top of the vacuole suspension. After centrifugation at 160*g* for 3 min at 4°C, the vacuoles were recovered in the upper phase. In these conditions the contamination by intact protoplasts never exceeded 1%.

Uptake Experiments

Uptake of [^{14}C]MACC and [^{14}C]malate was performed at 30°C in a reaction mixture containing 50 mM Tris-Mes buffer (pH 7), 426 mM sorbitol, 10 kBq [^{14}C]MACC (2.96 GBq·mmol^{-1}) or [^{14}C]malate (5.55 GBq·mmol^{-1}), 10 kBq [^{3}H]-H_2O either in the presence or in the absence of 5 mM MgATP and other additions as indicated in figures. The uptake was started by adding 200 μL of purified vacuole suspension (4.10^5 vacuoles) in the medium used for their isolation: 550 mM sorbitol, 10 mM Hepes-KOH (pH 7.0). At selected times, vacuoles were separated from the incubation medium by silicon layer floatation filtering according to the procedure of Martinoia *et al.* (19) slightly modified by Marigo *et al.* (17). Briefly, 100 μL of the reaction mixture containing the vacuoles were pipetted into a 400 μL polypropylene microcentrifuge tube, adjusted to 8.7% Nycodenz and overlayed first with 200 μL phenyl methyl silicon oil AP 100/AR 200 (100/83, v/v) and then with 50 μL of 2% Triton X-100. The subsequent centrifugation was performed as described previously (17). [^{3}H]H_2O was used to evaluate the internal volume of vacuoles (1–1.5 μL for 4.10^5 vacuoles) recovered after flotation. For each experiment, an assay with 10 kBq of [^{14}C]-dextran-carboxyl (44 MBq·g^{-1}), a nonpermeant agent, was made in order to estimate contaminations by the medium migrating with the vacuoles. The results were expressed either as pmoles MACC or malate taken up by 10^6 vacuoles or as the ratio C_i/C_e of internal and external [^{14}C]MACC concentration. Each experiment was made in duplicate.

Chemicals and Radiochemicals

The following compounds were of commercial origin: Caylase 345 was purchased from Cayla (Toulouse, France) and Pectolyase Y23 from Seishim Pharmaceutical Co (Chiba-Ken, Japan); Nycodenz was from Nyegaard & Co (Oslo, Norway), silicon oils AP 100 and AR 200 from Waker Chemie (Munchen, FRG) and [^{14}C]dextran-carboxyl from NEN Research Products). MACC was synthesized chemically according to the method of Satoh and Esashi (29). [2,3-^{14}C]ACC and [^{3}H]H_2O were from CEA (France) and *N*-malonyl [2,3-^{14}C]ACC was prepared biologically by feeding mungbeans hypocotyls with [2,3-^{14}C]ACC as described by Amrhein *et al.* (1) for buckwheat hypocotyls. The labeled MACC formed was purified by anion exchange and paper chromatography (11). Malonyl-D-Tryptophan and gramicidin were generously given by Professor Yang (University of California, Davis) and Professor Laneelle (University of Toulouse, France), respectively. All other chemicals were purchased from Sigma.

RESULTS

Time Course and Energy Dependence of MACC Uptake

The uptake of MACC was stimulated by *ca.* fourfold by 5 mM MgATP (Fig. 1). It increased rapidly until 45 min and slowed down thereafter. After only 30 min of incubation in the presence of MgATP, the MACC concentration became higher in the vacuole than in the external medium, indicating an accumulation against a concentration gradient. The addition of 300 μM PPi had no significant effect on MACC uptake.

Influence of pH on MACC Uptake

MACC transport into the vacuole was pH dependent in the presence of MgATP with an optimum between 6.5 and 7 (Fig. 2). In the absence of MgATP, the pH dependence is less pronounced with only small variations.

Effects of Temperature on MACC Uptake

Under energized conditions an optimum temperature for MACC uptake was observed at 30°C (Fig. 3). Arrhenius plots

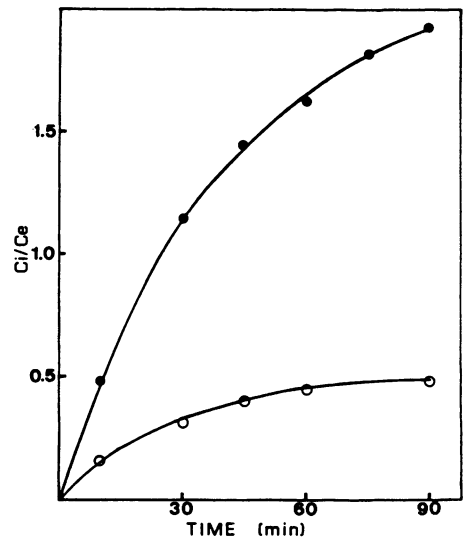

Figure 1. Time course of [^{14}C]MACC uptake by vacuoles isolated from *C. roseus* protoplasts. Aliquots of 200 μL of vacuole suspension (4.10^5 vacuoles in the isolation medium) were incubated at 30°C in a standard reaction mixture containing 50 mM Tris-Mes buffer (pH 7), 426 mM sorbitol, 10 kBq [^{14}C]MACC (2.96 GBq·mmol^{-1}) and 10 kBq [^{3}H]H_2O. C_i/C_e represents the ratio of internal *versus* external [^{14}C]-MACC concentration. At selected times, the reaction was stopped by centrifugation of 100 μL aliquots as described in "Material and Methods." External contaminations were estimated by the [^{14}C] dextran-carboxyl method and were subtracted from each uptake value. Without MgATP (○); in the presence of 5 mM MgATP (●).

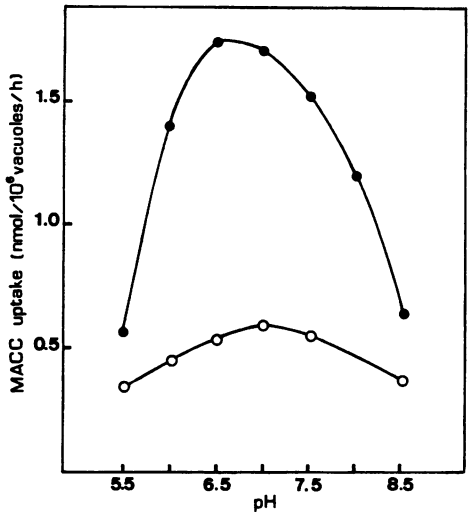

Figure 2. pH dependence of [^{14}C]MACC uptake into vacuoles. Aliquots of 200 μL of vacuole suspension (4.10^5 vacuoles) were incubated for 30 min at 30°C in 100 mM Tris-Mes buffer adjusted to the indicated pH values. All other experimental conditions and symbols are the same as in Figure 1.

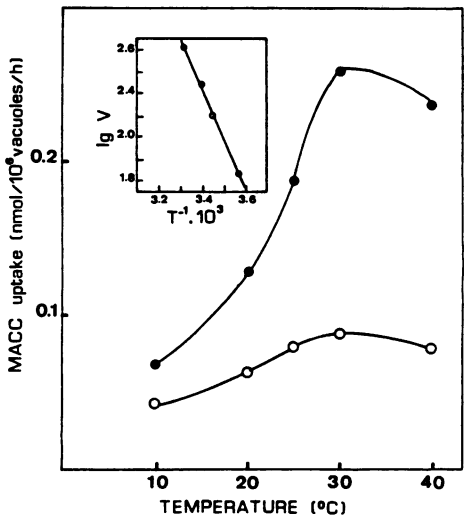

Figure 3. Temperature dependence of [^{14}C]MACC uptake into isolated vacuoles. Vacuoles were preincubated at the indicated temperatures for 10 min before addition of [^{14}C]MACC. Uptake experiments were carried out with and without 5 mM MgATP and stopped after 30 min incubation. All other experimental conditions and symbols are the same as in figure 1.

gave activation energies of 62 kJ · mol^{-1}. MACC transport into nonenergized vacuoles was weak and varied slightly with temperature. The uptake experiments were subsequently carried out at 30°C and at pH 7.

MACC Concentration Dependence of MACC Uptake

The uptake of MACC in the presence of MgATP was clearly saturable and the K_m value was estimated at 1.9 mM by Lineweaver-Burk plot (Fig. 4). The uptake in the absence of MgATP was too low to provide reliable K_m values.

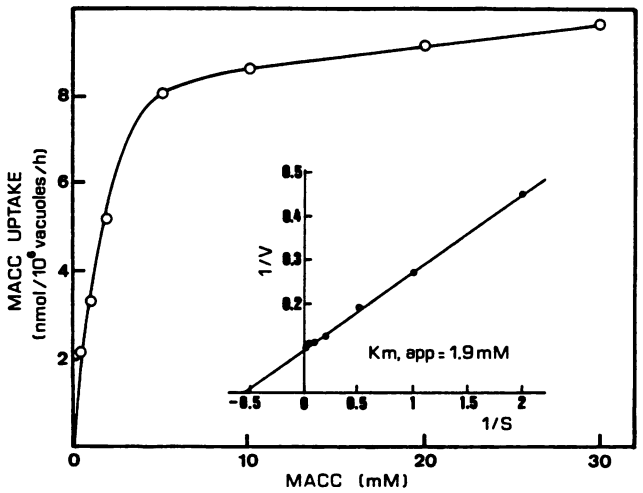

Figure 4. Concentration dependence of [^{14}C]MACC uptake into isolated vacuoles. The incubation was carried out for 30 min in the presence of 5 mM MgATP in the same conditions as described in Figure 1.

Table I. *Sensitivity to Inhibitors of MACC Uptake into Isolated Vacuoles*

Vacuoles were preincubated for 5 min with inhibitors before addition of [^{14}C]MACC and MgATP. The reaction was stopped after 1 h incubation by centrifugation of 100 μL aliquots as described in Figure 1. Values were expressed in percent of maximum [^{14}C]MACC uptake of control. Each value represents the means of three independent replicates.

Effectors	[^{14}C]MACC Uptake
	% of max. activity
Control	100
CCCP (12.5 μM)	27
Gramicidin (2 μM)	17
Benzylamine (10 mM)	16
DCCD (25 μM)	48
Valinomycin (10 μM)	33
SCN Na (8 mM)	11
Na Mes (8 mM)	113
K Mes (100 mM)	60

Determination of Driving Force Involved in MACC Uptake

Since MACC uptake was MgATP dependent, it was predictable that a proton motive force generated by tonoplastic ATPase was involved in this uptake. Table I shows that several effectors known to inhibit ATPase and/or to collapse H^+ electrochemical gradient (DCCD, CCCP, gramicidin and benzylamine) all reduced MACC transfer into the vacuole, DCCD being the least effective. On the other hand, SCN^- and valinomycin, reported to collapse specifically the membrane potential, also greatly affected MACC transport. It was verified that cations like Na^+ (8 mM), Ca^{2+} (3 mM), Mg^{2+} (5 mM) had no significant effect while K^+ (100 mM) inhibited MACC uptake by 40%.

Substrate Specificity of MACC Uptake

Table II shows that none of the MACC moities taken separately (ACC and malonate at 10 mM concentration) re-

Table II. *Structural Analogs Inhibition of [^{14}C]MACC and [^{14}C] Malate Uptake*

All inhibitors were supplemented to the reaction mixture containing $4 \cdot 10^5$ vacuoles and 5 mM MgATP 30 min before adding [^{14}C]MACC or [^{14}C]malate. Effectors were used at 20 mM except MACC, D-tryptophan, and L-tryptophan which were at 10 mM. For other experimental conditions see legend of Figure 1. Values were expressed in percent inhibition of the control activity and represent the mean of two independent experiments.

Effectors	Inhibition of Uptake	
	[^{14}C]MACC[a]	[^{14}C]Malate[b]
	% of max. activity	
ACC	6	ND[c]
Malonate	4	47
Malate	0	91
Malonyl-D-tryptophan	84	ND
Malonyl-ACC	89	1
D-Tryptophan	0	ND
L-Tryptophan	5	ND

[a] Values of 100% represents 167 pmol·10^6 vacuoles^{-1}·h^{-1}. [b] Value of 100% represents 82 pmol·10^6 vacuoles^{-1}·h^{-1}. [c] ND, not determined.

Table III. *Effect of Protein Binding Inhibitors*

Inhibitors were supplemented to vacuoles 5 min before adding [^{14}C]MACC. Uptake experiments were carried out without MgATP for 1 h. For other experimental conditions, see legend to Figure 1. Values represent the mean of two replicates.

Inhibitors	[^{14}C]MACC Uptake	Inhibition
	pmol·10^6 vacuoles^{-1}·h^{-1}	% of max. activity
Control	54	
Dansyl-chloride (200 μM)	34	39
NEM[a] (100 μM)	29	45
DIDS[b] (50 μM)	19	65
DCCD (25 μM)	25	55

[a] *N*-Ethylmaleimide. [b] 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid.

duced [^{14}C]MACC uptake (less than 7% inhibition) while unlabeled MACC and malonyl D-tryptophan (10 mM) acted as very effective competitors (89 and 84% inhibition, respectively). Malate, D-tryptophan, and L-tryptophan had no competitive effect. Malate was also taken up by these vacuoles. However this uptake was not affected by the addition of 10 mM MACC while it was reduced by 47% by malonate (Table II).

Effect of Protein Binding Inhibitors

The possible involvement of a protein entity in MACC transport was tested by applying protein-binding inhibitors in the absence of MgATP in order to avoid any interference of H^+-ATPase inhibition. *N*-Ethylmaleimide (100 μM) and dansyl-chloride (200 μM), reagents blocking SH- and OH-groups respectively, lowered MACC transport activity by 45 and 36%, respectively (Table III). 4,4′Diisothiocyanatostilbene-2,2′-disulfonic acid (50 μM), a compound blocking NH_2-groups, also known as an anionic channel inhibitor, was even more effective (65% inhibition).

DISCUSSION

The results reported in this paper demonstrate the occurence of MACC energy-dependent transport across the tonoplast of isolated vacuoles from *Catharanthus roseus* cells. They are in agreement with recent observations made by Tophof *et al.* (31) on isolated barley and wheat vacuoles. This transport is stimulated by MgATP as in the case of several other organic molecules like malate (17, 19), alkaloids (8) various sugars (30, 33), and amino acids (12). In a few cases, no evidence has been shown for a stimulating effect of ATP (7, 8). The occurence of an active transport had been widely questioned by Boller and Wiemken (4), because of the absence of rigorous demonstration of a net accumulation into the vacuole directly dependent upon energized transport. In our system, thanks to the calculation of C_i/C_e ratio we have been able to show that the rate of MACC uptake is extremely low in the absence of MgATP and no net accumulation occurs into the vacuole. By contrast, the transport is stimulated in the presence of MgATP, and MACC accumulates against a concentration gradient ($C_i/C_e > 1$). The involvement of an ATPase in MACC uptake is demonstrated by the sharp inhibitory effect of DCCD, a potent inhibitor of proton channels (2). Furthermore, the optimum pH (7.0) for the ATP-dependent transport of MACC is very similar to that of the ATPase of *C. roseus* (our unpublished data). Pyrophosphatase, another H^+ translocating enzyme which was found to be present on the tonoplast of the same material (9) and to stimulate malate uptake (17), has no effect on MACC uptake, probably because of the low electrochemical proton gradient generated by this system (17). Beyond the stimulatory effect of ATPase, another indication for a pH dependent MACC transport is the inhibitory effect of various compounds which decrease the electrochemical proton gradient (CCCP, gramicidin, benzylamine). Addition of high levels of thiocyanate, known to collapse membrane potential without affecting pH gradient (14), dramatically inhibited MACC uptake suggesting that membrane potential rather than pH gradient is directly involved. This data suggests that like other anions (14, 24) MACC transport is also driven by the inside positive membrane potential generated by the H^+ pumping ATPase. The diffusibility of K^+ seems also to play a part in this displacement since valinomycin, a K^+ ionophore, also affects the MACC transport. Two different mechanisms have been described for the transport accross the tonoplast, ionic channels identified by the patch-clamp technique (10) and protein carriers characterized by specific kinetic parameters. Our study clearly shows that MACC uptake as a function of MACC concentration is saturable with a low K_m of 2 mM. Moreover the transport system described in this work has a specificity for MACC and malonyl-D-tryptophan. It is noteworthy that the carrier recognizes specifically the malonylated compounds resulting from the malonyl transferase activity (15, 20). None of the two MACC moities taken separately (ACC and malonate) reduced MACC uptake. Similar results were reported by Matern *et al.* (21) for the uptake of apigenin 7-*O*-(6-*O*-malonylglucoside) into parsley vacuoles. It remains

to be determined whether such malonylated flavonoids could also be recognized by the MACC transport system. Another indication for the tight specificity of transport proceed from the fact that no interference has been shown with the malate-carrier also localized in the same membrane (17). Furthermore malonate reduces malate uptake (17) when it was ineffective on MACC transport. Our experiments indeed show that neither MACC transport was affected by malate nor malate transport was inhibited by MACC. These data are in opposition with the results reported by Tophof *et al.* (31) in other materials, indicating an inhibition of malate transport by MACC with a high K_i of 5 to 8 mM as compared to a low K_m of 1 mM for malate. In this case however no study has been done on the effect of malate or other organic acids also transported by the malate-carrier on MACC transport. In our study, an optimum pH (around 7) and temperature (30°C) for MACC uptake was observed associated with a value for the activation energy (62 $kJ \cdot mol^{-1}$) that argues in favour of catalyzed transport. The direct involvement of a protein in MACC transport is also suggested by the inhibitory effect of reagents that block OH—, SH—, or NH_2— groups. These compounds were used in the absence of MgATP, therefore avoiding any interference with ATPases inhibition. All these data strongly indicate that MACC uptake into vacuoles is mediated by a specific system of transport sites and provide evidence of the existence of a protein carrier for the MACC transport across the tonoplast of *C. roseus*.

Once in the vacuole, [^{14}C]MACC cannot be released either by collapsing membrane potential (or pH gradient) or by adding high external concentrations of unlabelled molecule (data not shown). Similar investigations have led to the demonstration of a vacuolar efflux in the case of other organic molecules such as alkaloids (8). Such discrepancy could come from an isotopic dilution of [^{14}C]MACC in the MACC-rich vacuolar sap, thus masking the efflux of the labelled compound. However, the absence of MACC efflux through the tonoplast is a confirmation of our previous results showing the sequestration of MACC upon washing of *Acer pseudoplatanus* cells (6). Some cases of definitive sequestration have been described in which the compounds undergo conformational changes (25) or ion-trap mechanism (22). However, MACC is not capable of symetry changes and therefore trapping cannot result from conformational changes of the transported substance. An ion-trap mechanism also seems to be inconceivable because at the pH used for uptake, MACC is predominantly in the $HMACC^-$ form, since we have determined that the various values for pK are 2.8, 5.2 and 11.9. Once in the vacuole the change in pH is not sufficient to result in a sequestration of the molecule in the H_2MACC form. However this hypothesis implies that the protonated form is not permeant. The most probable explanation is that MACC becomes bound to cations, a mechanism previously described (18) for citrate accumulation. The absence of MACC efflux may be due to the absence of an internal binding site, thus giving arguments for a polarized transport.

Liu *et al.* (15), demonstrated that the malonylation of ACC and D-amino acids are intimately interrelated. It was recently reported (26) that many plants respond to drought stress by D-tryptophan formation followed by its *N*-malonylation. It was suggested that the physiological significance of *N*-malonylation is to inactive D-amino acids which might otherwise be toxic to plants (28). The transport and the sequestration of these amino acid derivatives into the vacuole space, described in this work, can therefore be considered as contributing to a detoxification mechanism of plant cells by withdrawing these molecules from the cytosol.

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