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Intracellular Sites of Synthesis and Storage of 1-(Malonylamino)cyclopropane-1-Carboxylic Acid in *Acer pseudoplatanus* Cells[1]

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ABSTRACT

Vacuoles were isolated from *Acer pseudoplatanus* cells that were incubated with [^{14}C]1-aminocyclopropane-1-carboxylic acid (ACC). The kinetics of [^{14}C]1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) formation are consistent with the interpretation that MACC is synthesized in the cytosol, transported through the tonoplast, and accumulated in the vacuole. Twenty hours after chasing the labeled ACC with unlabeled ACC and adding 1 millimolar unlabeled MACC, all the [^{14}C] MACC synthesized is located in the vacuole. Whole cells preloaded with [^{14}C]MACC and then submitted to a continuous washing out, readily release their cytosolic MACC until complete exhaustion. The half-time of MACC efflux from the cytosol, calculated by the technique of compartmental analysis, is about 22 minutes. In contrast, vacuolar MACC remains sequestered within the vacuole. The transport of labeled MACC into the vacuole is stimulated by the presence of unlabeled MACC in the suspension medium, probably as a result of a reduced efflux of the labeled MACC from the cytosol into the suspending medium.

The immediate precursor of ethylene, ACC[2] (1, 15), has been found to be conjugated into MACC in all plant tissues so far examined (3, 4, 11, 12). Since the rate of ethylene production seems to be controlled generally by the ACC levels in the cell (13, 22) and malonylation of ACC can regulate ACC levels, malonylation could play an important part in controlling ethylene production (14, 23). It has been previously demonstrated that MACC is either wholly (6) or predominantly (5) localized in the vacuole. However, its site(s) of synthesis and its movements between the various cell compartments have not been studied, although it has been suggested that malonylation of secondary compounds could occur at the tonoplast in association with the vacuolar deposition of these compounds (17).

Procedures for determining the site of synthesis and storage of metabolites in plant cells have been developed (18). These procedures require that protoplasts are preloaded with the labelled precursor of the compound under study, and at specified intervals protoplasts are sampled, pure vacuoles are prepared from an aliquot of these protoplasts, and radioactivity of the compound in protoplasts and vacuoles is determined. In the present study, we have used a method (2) in which a polybase-induced lysis of the plasmalemma followed by centrifugation has allowed a very fast release of the vacuole (within a few seconds). It is therefore suitable to measure the time course of synthesis of MACC in the protoplast and its possible transport across the tonoplast. *Acer pseudoplatanus* cells have been used to determine the intracellular site(s) of MACC synthesis, to demonstrate its sequestration in the vacuole and to establish, by the technique of compartmental analysis, the relative permeability of the tonoplast and plasmalemma to MACC.

MATERIALS AND METHODS

Cell Cultures, Preparation of Protoplasts and Vacuoles. *Acer pseudoplatanus* cells were grown as previously described (2). Protoplasts were isolated according to the procedure of Alibert *et al.* (2) with the following modifications: 8 g of cells were harvested during the exponential growth phase, *i.e.* between 6 and 9 d after transfer into new medium. After rinsing with fresh medium through a Büchner-type funnel, cells were placed in a Petri dish in 20 mL of 25 mM Mes-Tris buffer (pH 5.5) in 0.7 M mannitol (medium A) containing 2% (w/v) caylase and 0.1% (w/v) pectolyase. The digestion was performed at 36°C for 35 min with gentle shaking (44 oscillations min^{-1}). After filtration through a 25-μm nylon net, the released protoplasts were washed three times in 2 mL of 25 mM Mes-Tris buffer (pH 6.5) containing 0.7 M mannitol (medium B) and centrifuged at 150*g* for 90 s. The washed protoplasts were then diluted in medium B to a final concentration of 1.5×10^6 to 2.0×10^6 protoplasts mL^{-1}.

Vacuoles were released from the protoplasts by the procedure reported in (2), combining the polybase-induced lysis of plasmalemma and centrifugation. The gradient was slightly modified as follows (from bottom to top): 2 mL dextran sulfate (3 mg·ml^{-1}) in 10% Ficoll dissolved in medium B, 2 mL dextran sulfate (1mg·mL^{-1}) in 5% Ficoll dissolved in medium B and 6 mL DEAE dextran (6 mg·mL^{-1}) in 2.5% Ficoll dissolved in medium B. Vacuoles and protoplasts were counted using a Fuchs-Rosenthal hemacytometer.

Vacuoles from cells previously washed during a 72-h period behaved differently during the isolation procedure; they were collected at the interface of 0 to 2.5% Ficoll instead of 5 to 10% as for standard cells.

Chemical and Radiochemicals. Caylase 345 was purchased from Cayla (Toulouse, France) and pectolyase Y23 from Seishin Pharmaceutical Co. (Chiba-Ken, Japan). MACC was synthesized chemically according to the method of Satoh and Esashi (21). [2,3-^{14}C]ACC (2.96 GBq $mmol^{-1}$) was kindly prepared by CEA (France) and *N*-malonyl[2,3-^{14}C]ACC was prepared biologically by feeding mungbeans hypocotyls with [2,3-^{14}C]ACC as de-

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[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid: α-AIB, α-aminoisobutyric acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid.

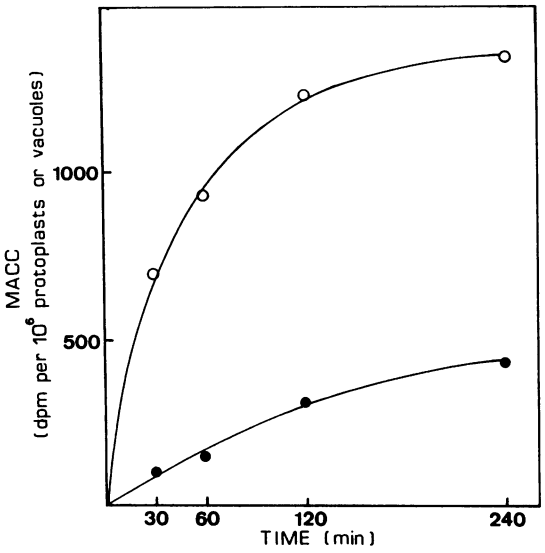

FIG. 1. Kinetics of MACC formation in protoplasts (○) and corresponding vacuoles (●) after feeding protoplasts with 0.125 mM [^{14}C]ACC (2960 MBq·mmol^{-1}).

scribed by Amrhein *et al.* (3) for buckwheat hypocotyls. The labeled MACC formed was purified by anion exchange and paper chromatography (11). The other chemicals were as in Alibert *et al.* (2).

Extraction and Estimation of [^{14}C]MACC. MACC was extracted under reflux with boiling 70% ethanol for 15 min after sonication of protoplasts or lysing of vacuoles by freezing and thawing. After removal of ethanol under vacuum at 40°C, MACC was purified by anion exchange chromatography (1 mL Dowex 1 × 8, $HCOO^-$ form, 100–200 mesh) and eluted with 15 mL of 2 N formic acid. After evaporation to dryness, the residue was dissolved in water and radioactivity was determined by scintillation counting (LKB 1215).

Calculation of MACC Contents in Protoplast and Vacuole. The MACC content of the protoplasts was expressed on the basis of the protoplast number obtained by direct counting. The vacuolar MACC content was calculated on the basis of α-mannosidase activity, a vacuolar marker (18).

Feeding Protoplasts with [2,3-^{14}C]ACC. Two mL of protoplast suspension (20 × 10^6 protoplasts mL^{-1} in medium B) were added to 27-mL glass vials containing 0.125 mM [^{14}C]ACC (2.96 GBq·mmol^{-1}) and incubated with shaking at 25°C. At selected times, protoplasts were sedimented by centrifugation and the supernatant retained. The protoplasts were then washed gently twice with medium B, and vacuoles were isolated as described above.

Washout Studies. The technique of compartmental analysis of radioisotope elution was used to determine the rate constant (K) and half-time ($t_{1/2}$) for passive MACC efflux from the various cell compartments (16, 19, 20). Six mL of 13-d-old cells were incubated in the presence of 1 mM [^{14}C]MACC (8.8 MBq·mmol^{-1}). After incubation for 5 or 72 h in the same conditions as the stock cells were cultured (2) the external medium was removed by decanting. A portion of cells (2 mL) was placed in a small column and was continuously eluted with new culture medium by means of a peristaltic pump at a rate of 0.4 mL per min. The flow-through medium was collected as 0.2-mL fractions during the first 25 min, and as 2-mL fractions during the following 4 h 35 min, and counted for radioactivity. After the washout, cells were collected and prepared as previously described for the estimation of remaining [^{14}C]MACC.

RESULTS

Kinetics of MACC Formation in Protoplast and Vacuole. The kinetics of [^{14}C]MACC formation were followed during a 4-h period after feeding protoplasts with [^{14}C]ACC. Figure 1 shows that the level of MACC formed increased rapidly in the protoplasts during the first 2 h and then reached a constant level. Most of the MACC synthesized was present in the extravacuolar compartment. The vacuolar content of MACC increased relatively slowly with time and always remained low.

Kinetics of Transport of MACC into the Vacuole. In order to follow the transport of the [^{14}C]MACC formed, protoplasts were first incubated for 6 h in the presence of labeled ACC, the protoplasts were sedimented by centrifugation, the supernatant discarded, and the protoplasts resuspended in a similar medium but containing 12.5 mM unlabeled ACC in order to abolish effectively the formation of labeled MACC. Under these conditions, about 50% of the MACC formed was recovered in the vacuole fraction after 20 h (Fig. 2A), thus indicating a slow but continuous transport from the cytosol into the vacuole. By providing 1 mM unlabeled MACC together with 12.5 mM unlabeled ACC the transport of MACC into the vacuole seemed to be promoted since after 19 h and 24 h 70% and 100% of the [^{14}C]MACC had entered the vacuole, respectively (Fig. 2B). Confirmation of the influence of exogenous MACC on the transport of MACC into the vacuoles was made by using increasing amounts of nonradioactive MACC and measuring the labeled MACC content in vacuole fraction after 19 h (Table I). The percentage of MACC in the vacuole increased from 63% in the control to 73%, 85%, and 100% for MACC supplied at concentrations of 1, 5, and 10 mM, respectively. The diffusion of [^{14}C]MACC into the external medium decreased with increasing concentrations of unlabeled MACC, while the total amount of labeled MACC synthesized during the incubation period re-

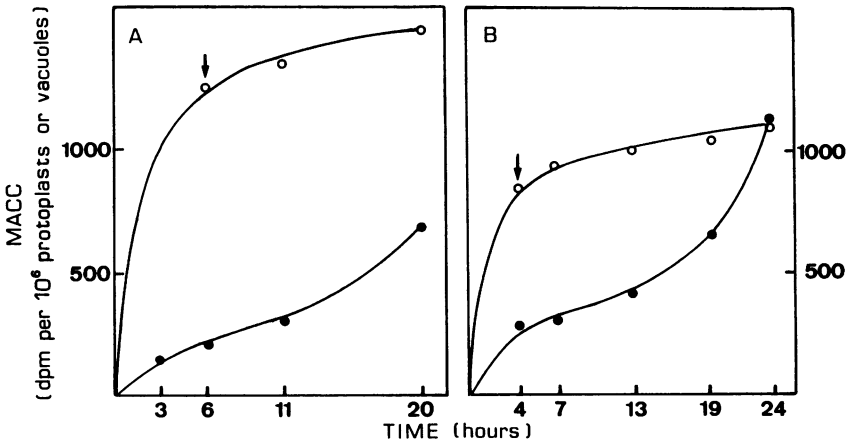

FIG. 2. Kinetics of transport of [^{14}C]MACC in the vacuole. Two ml of protoplast suspension (20 × 10^6 protoplasts ml^{-1}) were first incubated in the presence of 0.125 mM [^{14}C]ACC (2960 MBq·mmol^{-1}), then the supernatant was removed by centrifugation and replaced by 2 ml of new medium containing: A, 12.5 mM unlabeled ACC at arrow (6 h); B, 12.5 mM unlabeled ACC and 1 mM unlabeled MACC at arrow (4 h). At selected times, radioactive MACC was counted in protoplasts (○) and corresponding vacuoles (●).

Table I. *Effect of Supplying External Unlabeled MACC on the Intracellular Distribution of [^{14}C]MACC Synthesized in Situ*

Protoplasts were incubated in the presence of [^{14}C]ACC (0.125 mM, 2.96 GBq·mmol^{-1}). After 4 h, the supernatant was separated by centrifugation and replaced with new medium containing 12.5 mM unlabeled ACC and increasing concentrations of unlabeled MACC. The levels of [^{14}C]MACC were determined in the whole suspension, and in protoplasts and corresponding vacuoles after incubation for another 15 h.

Unlabeled MACC Supplied	[^{14}C]MACC[a]		[^{14}C]MACC in Vacuole	[^{14}C]MACC in Whole Suspension[b]	[^{14}C]MACC in External Medium[b]
mM	*dpm (10^6 protoplasts)$^{-1}$*	*dpm (vacuoles from 10^6 protoplasts)$^{-1}$*	*%*	*dpm*	*dpm*
0	730	460	63	1450	720
1	770	570	74		
5	830	800	96	1370	540
10	960	980	102	1360	400

[a] Values corresponding to the distribution of [^{14}C]MACC after washing protoplasts with medium B at the end of the 15 h incubation period and isolation of vacuoles as described in "Material and Methods." [b] Values corresponding to [^{14}C]MACC synthesized in the whole protoplast suspension or in the external medium corresponding to 10^6 protoplasts with no washing after 15 h.

Table II. *Intracellular Distribution of [^{14}C]MACC after Preincubating Acer Cells with [^{14}C]MACC for Different Times*

Cells were preincubated with 1 mM [^{14}C]MACC (8.8 MBq·mmol^{-1}) for the specified times, washed as indicated, and then protoplasts and vacuoles were prepared from the washed cells to determine the intracellular distribution of labeled MACC.

Time of Preincubation with [^{14}C] MACC	[^{14}C]MACC in Cells		[^{14}C]MACC in Cells after Washing	Intracellular Distribution of [^{14}C]MACC		[^{14}C]MACC in Vacuoles
	Before washing	After washing				
h	*dpm (10^6 cell)$^{-1}$*		*percent*	*dpm (10^6 protoplasts)$^{-1}$*	*dpm (vacuoles from 10^6 protoplasts)$^{-1}$*	*percent*
5	12,560	250	2	ND[a]	ND	ND
72	11,510	3,010	26	2,980	3,130	104

[a] ND = not determined.

mained largely unchanged (Table I). A conversion of MACC to ACC was never observed.

Kinetics of Efflux of Labeled MACC. Cells collected at the stationary phase were brought to high density (packed cell volume of 0.5 ml cells mL^{-1}) in order to greatly reduce cell division. They were preloaded with [^{14}C]MACC, decanted, and then submitted to continuous washing. At the end of the wash-out period, 2% (after 5 h preloading) and 26% (after 72 h preloading) of the radioactivity was retained inside the cells and all the cellular [^{14}C]MACC was recovered in the vacuole (Table II). From counts of the radioactivity remaining in the cells at the end of the elution and from radioactivity in each of the washings, an efflux curve was constructed (Fig. 3). The final line obtained, either on the initial curve or after subtraction of the vacuolar and cytosolic component, has a slope corresponding to the rate constant (K = ln dpm/time) of [^{14}C]MACC efflux from the vacuole (Fig. 3A), cytosol (Fig. 3B), and free space (Fig. 3C) compartments. The rate constant (K) and the half-time for loss of labeled MACC ($t_{1/2} = \ln 2/K$) were calculated for each compartment by graphical analysis. Elution profiles of cells preloaded for 72 h indicated a complete cessation of the efflux after 75 min (Fig. 3A). The rate constant of efflux from the slow exchange compartment (vacuole) was therefore 0 and the $t_{1/2}$ infinite (Table III). By contrast, cytosolic and apoplastic MACC effluxed very readily (Fig. 3, B and C) with rate constants of 0.031 min^{-1} and 1.44 min^{-1} and half-times of 22 min and 0.48 min, respectively (Table III). The eluted radioactive material was confirmed to be MACC by its retention on anionic exchange resin.

DISCUSSION

Our present results show that after feeding protoplasts with [^{14}C]ACC for a short incubation time, most of the newly synthesized labeled MACC was located in the cytosol and slowly transfered into the vacuole, thus demonstrating that the cytosol is the site for MACC synthesis. The cytosol is believed to be the site of ACC synthesis (22) and thus a cytosol located ACC-malonyltransferase could regulate directly the levels of newly-synthesized ACC without an intervening transmembrane transport of ACC. Also, the present finding of a cytosolic location for the malonylation of ACC is incompatible with the previous suggestion (17) that malonyltransferases may have a role in vacuolar transport.

After chasing [^{14}C]ACC with unlabeled ACC and incubating for a longer period of time, the bulk of the [^{14}C]MACC was recovered in the vacuole. When the external concentration of nonradioactive MACC was increased, the relatively slow [^{14}C] MACC transport was accelerated. This phenomenon can be interpreted on the basis that export of [^{14}C]MACC into the

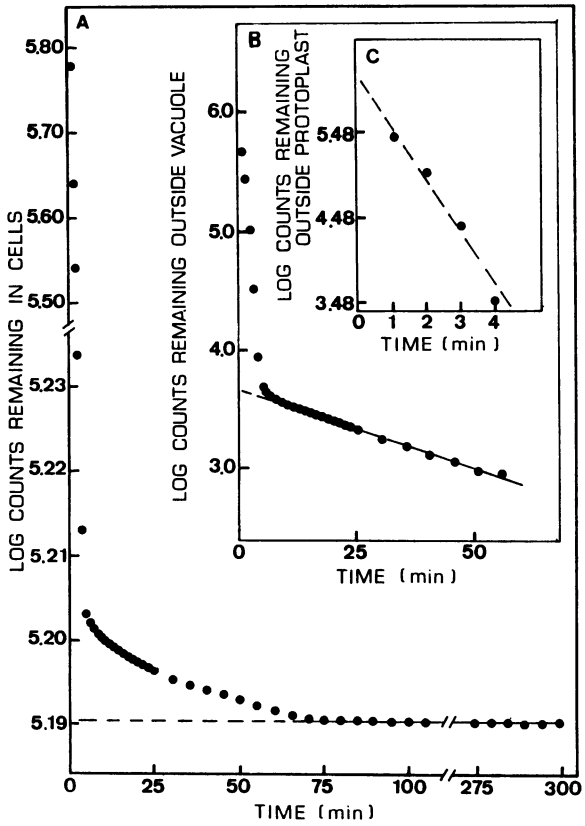

FIG. 3. Semilog plots corresponding to the time course of the reduction in the amount of [^{14}C]MACC during washing in the various compartments of *A. pseudoplatanus* cells. A, Log counts remaining in whole cells; B, log counts remaining outside vacuole after subtraction of the vacuolar component (value corresponding to the intercept with time 0 of final line of A); C, log counts remaining outside the protoplast after subtraction of the cytosolic component (value corresponding to the intercept with time 0 of the final line of B).

Table III. *Rate Constant and Half-Time for [^{14}C]MACC Efflux*

Cells (13 d old) were incubated in the presence of [^{14}C]MACC (8.8 MBq·mmol^{-1}) for 72 h. Then the supernatant was removed by centrifugation and an aliquot (2 mL) of cells was submitted to continuous elution as described in "Materials and Methods."

Compartment	Rate Constant	Half-Time
	min^{-1}	*min*
Vacuole	0	
Cytosol	3.1×10^{-2}	22
Free space	1.44	0.48

extraprotoplastic medium was reduced, resulting in a higher retention of [^{14}C]MACC in the cytosol. Thus, the transport of MACC into the vacuole is governed not only by the concentration of MACC in cytosol but also by that outside the plasmalemma.

The question of the possible retention of MACC inside the vacuole could not be adressed by washing out MACC from isolated vacuoles because of their extreme fragility. For this reason we used the method of compartmental analysis of intact cells (16, 19, 20).

The efflux of MACC from the free space and cytosol compartments occurred with rate constants of 1.44 and 0.031 min^{-1} (Table III). These rate constants were similar to the rate constants determined for α-AIB efflux from the free space and cytosol of tomato tissues (20). All the MACC retained in the cells after washing was present in the vacuole indicating a complete sequestration in this compartment, as has been observed for hormone conjugates and secondary compounds (8).

The demonstration that MACC can easily diffuse from the cytosol through the plasmalemma indicates that caution must be taken in the limitation of MACC efflux during washing of cells or protoplasts. In our kinetics studies, [^{14}C]MACC diffusion from the cytosol was reduced by using high protoplast densities (20×10^6 protoplasts mL^{-1}), and/or by supplementing the external medium with unlabeled MACC. However, experiments devoted to the estimation of MACC concentration in the various compartments of the cells require severe purification and washing of protoplasts. Under these conditions it is likely that there is a rapid efflux of MACC, and therefore its concentration in the cytosol of protoplasts may have been underestimated. From previous experiments (6, 7) we had observed that MACC was entirely localized in the vacuole. In view of the present findings it is now apparent that the previously observed absence of MACC from the cytosol may have been due to a loss by diffusion across the plasmalemma. When MACC has been reported to be present in the cytosol (5), the tissues had been previously stressed, and consequently they probably had a much higher malonylation capacity than the *Acer* cells used in the present work. Moreover, it was noted (5) that the fractionation of freeze-stopped material in nonaqueous media (10), by avoiding efflux of metabolites, gave higher values for the cytosolic level of MACC.

Our data also suggest that MACC by migrating through the plasmalemma can be transported into other plant parts, especially when it is overproduced as under stress conditions (9).

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