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Subcellular Localization of 2-(β-D-Glucosyloxy)-Cinnamic Acids and the Related β-glucosidase in Leaves of *Melilotus alba* Desr.[1]

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ABSTRACT

The distribution of the glucosides of *trans-* and *cis*-2-hydroxy cinnamic acid and of the β-glucosidase which hydrolyzes the latter glucoside was examined in preparations of epidermal and mesophyll tissue obtained from leaves of sweet clover (*Melilotus alba* Desr.). The concentrations of glucosides in the two tissues were about equal when compared on the basis of fresh or dry weight. Inasmuch as the epidermal layers account for no more than 10% of the leaf volume, the mesophyll tissue contains 90% or more of the glucosides. Vacuoles isolated from mesophyll protoplasts contained all of the glucosides present initially in the protoplasts.

The specific activities of the β-glucosidase in the two tissues were also similar; thus, most of the enzyme is contained in mesophyll tissue. However, the amount of enzyme in mesophyll protoplast extracts amounts to only 1 to 2% of the activity present in leaf homogenates when chlorophyll was the basis for comparison. (This small amount of coumarin-β-glucosidase present in protoplasts is not associated with chlorophyll-containing fractions.) In contrast, 90% of the uridine diphosphate glucose-*o*-coumaric acid glucosyl transferase activity present in leaf homogenate was recoverable in protoplasts prepared from intact leaves. Such results indicate that most of the coumarin-β-glucosidase in *M. alba* leaves is located in the extracytoplasmic space. Only a small fraction (7%) of this extra cytoplasmic β-glucosidase was associated with individual cells or cell clusters isolated from clover leaves.

Two isomers of 2-(β-D-glucosyloxy)-cinnamic acid occur in leaves of sweet clover (*Melilotus alba*) in approximately equal amounts (12, 13). One is the glucoside of 2-hydroxy-*trans*-cinnamic acid (*o*-coumaric acid) or *o*-coumaryl glucoside. The other is the glucoside of 2-hydroxy-*cis*-cinnamic acid (coumarinic acid) or coumarinyl glucoside, also known as 'bound coumarin' (12). A β-glucosidase which also occurs in sweet clover leaves hydrolyzes the coumarinyl glucoside (bound coumarin) but is inactive towards the other glucoside (12, 13). When clover leaves are disrupted, the glucosidase liberates coumarinic acid, which spontaneously lactonizes and forms coumarin.

Recent studies have shown that dhurrin, another plant glucoside, and the enzymes catalyzing its decomposition are located in different cellular compartments (11). In this paper, we examine the localization of these two glucosides (referred to as the coumarin-glucosides) and the related β-glucosidase in leaves of *M. alba* using epidermal and mesophyll tissues, mesophyll protoplasts, and vacuoles isolated from mesophyll protoplasts.

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MATERIALS AND METHODS

Plant Materials. Only the youngest fully expanded leaves, from which the lower epidermis could be easily peeled, were employed in the experiments described in Tables I and II. They were harvested immediately before use from plants, *Melilotus alba* Desr. var. Spanish, grown in the green house or growth chamber.

Assay of *o*-Coumaric Acid and Two Related Glucosides. *o*-Coumaric acid and the two isomeric coumarin-glucosides were determined by the fluorometric method of Haskins and Gorz (5). Tissue samples (lower epidermis or mesophyll with upper epidermis attached) were ground in 95% ethanol in a mortar with a pestle and filtered through a weighed glass filter (Whatman GF/C). The filtrates from lower epidermis and mesophyll plus upper epidermis were made to 5 and 25 ml, respectively, with 95% ethanol, and their *A* at 649 nm and 665 nm was measured immediately to determine their Chl content (22). Five ml of each alcoholic filtrate was dried *in vacuo*, and 5.0 ml water were added to dissolve the residue. A 1-ml aliquot of the well-mixed aqueous extract was added to 9 ml 2.5 N NaOH in a Pyrex test tube. After mixing, two 1.0-ml aliquots of the alkaline solution were withdrawn, added to 9 ml water in a test tube, and saved for fluorometric analysis to determine any *o*-coumaric acid present. The alkaline solution remaining (8.0 ml) was autoclaved for 45 min at 120°C to hydrolyze the coumarin glucosides present in the original sample (12). The autoclaved solution was cooled, and 1-ml samples were diluted to 10 ml with water. These and the earlier nonautoclaved diluted samples were exposed to UV light (peak near 360 nm) for 15 min at a distance of approximately 15 cm from the filter to obtain an equilibrium mixture of the *cis-* and *trans*-isomers of 2-hydroxycinnamic acid. The fluorescence of these samples was then measured in Fluoro-colorimeter (American Instrument Company, Silver Spring, MD; J4-7439). A freshly prepared set of coumarin standards, including concentrations of 0.1, 0.25, 0.50, 0.75, and 1.0 μg of coumarin per ml in 0.5 N NaOH, was irradiated and read with each set of samples. The content of the two glucosides was calculated by subtracting the values of the nonautoclaved solutions from those of the autoclaved solutions.

Preparation of Crude Homogenates for Analysis of β-Glucosidase Activity. Leaf samples were frozen in liquid N_2, powdered using a mortar and pestle, and then added to 6 volumes of tissue weight of 50 mM sodium acetate buffer (pH 5.25) containing 10 mM sodium ascorbate. After further mixing, the homogenate was centrifuged for 30 min at 11,000*g*, and an aliquot (1 ml) of the supernatant solution was passed through a Sephadex G-25 column (5 × 1.5 cm) preequilibrated with 50 mM sodium acetate buffer (pH 5.25). The supernatant solution and the Sephadex G-25 effluent were used for enzyme assay.

Assay for β-Glucosidase. The β-glucoside of melilotic acid (2-hydroxyphenylpropanoic acid) was used as substrate, because

Table I. *Distribution of o-Coumaric Acid and Coumarin Glucosides in Leaves of M. alba*

	o-Coumaric Acid	Coumarin Glucosides	*o*-Coumaric Acid	Coumarin Glucosides
	µmol/g fresh wt		*µmol/g dry wt*	
Upper epidermal tissue	5.5	25.3	63	289
Mesophyll tissue with lower epidermis attached[a]	19.5	40.6	102	213
Mesophyll tissue with upper epidermis attached[a]	15.1	34.0	72	162
Lower epidermal tissue	8.6	30.1	62	216

[a] The epidermal tissue constituted no more than 6% of the sample (based on protein).

Table II. *Distribution of β-Glucosidase in the Leaves of M. alba*

The values in parentheses were obtained with the effluent from a Sephadex G-25 column. The figures in the table are the mean value for three experiments.

	β-Glucosidase Activity			Protein
	Protein	Fresh weight of tissue	Intact leaves	
	µmol/min·mg	*µmol/min·g*	*µmol/min·g*	*mg/g leaves*
Mesophyll tissue with upper epidermis attached	3.2 (3.6)	76.6	62.2	19.3
Lower epidermal tissue	2.7 (4.6)	16.0	3.7	1.25

assays involving coumarinyl glucoside are complicated by the facile light-catalyzed isomerization of the latter compound to *o*-coumaryl glucoside which is not hydrolyzed by the enzyme (12, 13). The melilotyl glucoside (m.p. 172–173°C) was prepared by hydrogenation of *o*-coumaryl glucoside in methanol with a catalyst of 5% platinum on charcoal. The *o*-coumaryl glucoside (m.p. 245–246°C) was synthesized according to Helferich and Lutzman (8).

The assay mixture consisted of 50 mM sodium acetate buffer (pH 5.25), 10 mM melilotyl glucoside, and the enzyme to be assayed in a final volume of 150 µl. The reaction mixture was incubated in a small test tube for 4 or 6 min at 37°C with shaking, after which 100 µl of 0.5 N H_2SO_4 were added to stop the reaction. H_2O (350 µl) was then added; after standing for 10 min, 100 µl diazotized *p*-nitroaniline solution (14, 20), 50 µl 20% NaOH in small drops with thorough mixing, and 500 µl H_2O were added in that order, and the samples were read at 490 nm against a standard curve of melilotic acid. Under the conditions of the assay, the amount of reaction was linear with time up to 8 min. The reaction was also proportional to the protein added up to 8.1 µg. Unless otherwise stated, 1 to 4 µg of enzyme protein was used.

Preparation of Subcellular Fractions. Leaf samples (1.5 g) were ground in a cold mortar with 10 ml of 50 mM phosphate buffer (pH 6.7) containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 10 mM sodium ascorbate to give a workable slurry, which was squeezed through 1 layer of Miracloth (Chicopee Mills, Inc., Milltown, NJ). To determine the subcellular localization of the β-glucosidase activity, the filtrate was subjected to differential centrifugation at 500*g* for 10 min, 11,000*g* for 10 min, and 100,000*g* for 1.5 h. Each particulate fraction obtained was washed once by resuspension in 10 mM phosphate buffer containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 5 mM sodium ascorbate and collected by resedimentation.

Preparation of Intact Chloroplasts from Protoplasts. The lower epidermis was carefully removed by peeling the youngest fully expanded leaves selected from a plant transferred 24 h before use to a dark room. This prevented the clumping of chloroplasts which occurred in the presence of starch. Samples of mesophyll with upper epidermis attached (1.1 g) were placed upon the surface of the digestion medium (40 ml) consisting of medium A, 3% (w/v) Cellulysin (Calbiochem), 0.75% (w/v) Macerase (Calbiochem), and 0.05% (w/v) BSA in a Petri dish. The sample was then evacuated quickly (40 mm Hg) with a vacuum pump; the air was readmitted, and the sample was digested for 2 h at 30°C in a shaking water bath at 45 oscillations/min. The contents of the Petri dish were then filtered through a 44-µm nylon net, and the released protoplasts were harvested by centrifugation of the filtrate at 100*g* for 3 min in a bench-top swinging-bucket centrifuge. The protoplast pellet was washed once using medium A and twice using medium B and finally resuspended in 1.8 ml of medium B. The protoplasts were then gently ruptured by drawing the suspension up and down 10 times through a 25-gauge needle using a 1-ml syringe. The lysate was filtered through two layers of 10-µm nylon net in a Swinnex-13 Millipore filter unit (Millipore, Bedford, MA), and an aliquot (0.9 ml) of the resultant suspension was then loaded onto the top of a linear 30 to 60% (w/w) sucrose gradient (40 ml) made up in medium C. The gradient was centrifuged for 45 min at 25,000 rpm in a Beckman SW 27 rotor using a Sorvall OTD-50 Ultracentrifuge at 4°C. Fractions (1.35 ml) were collected using the ISCO model 185 gradient fractionator (Instrumentation Specialities Co., Lincoln, NE). Each fraction was analyzed for β-glucosidase activity, Chl content, sucrose content, and NADPH-dependent trioseP dehydrogenase activity. NADPH-dependent trioseP dehydrogenase activity was measured by the method of Heber *et al.*, (7).

Preparation of Vacuoles from Protoplasts. Vacuoles were isolated as described from protoplasts prepared as reported by Boudet *et al.* (3). Such protoplasts (and vacuoles) were used in the studies described in Tables III to V. *o*-Coumaric acid:UDPG glucosyl transferase in these preparations was assayed as described previously (18).

Preparation of Isolated Cells. Isolated cells were obtained from mesophyll tissue by the procedure of Abravanel *et al.* (1).

Media. The following media were used for isolation of protoplasts and intact chloroplasts: medium A, 25 mM Mes-Tris buffer (pH 5.5) containing 0.7 M mannitol, 10 mM sodium ascorbate, and 0.5 mM $MgCl_2$; medium B, 20 mM Tricine-NaOH (pH 7.5) containing 0.7 M mannitol, 0.1 mM EDTA, and 1 mg/ml BSA; and medium C, 20 mM Tricine-NaOH (pH 7.5) containing 1 mg/ml BSA.

Protein and Chl Estimations. Protein was estimated by the method of Lowry *et al.* (15), after precipitation with TCA and dissolution in dilute base. BSA was used as a standard. Total Chl was determined by the method of Arnon (2).

RESULTS

Distribution of *o*-Coumaric Acid and the Coumarin Glucosides in Leaves. Table I shows that the upper epidermis, mesophyll, and lower epidermis of sweet clover leaves each contained *o*-coumaric acid mainly in the form of its two glucosides. The concentration of total *o*-coumaric acid (free acid plus glucosides) in the upper or lower epidermis was somewhat lower than that in mesophyll tissue, when compared on the basis of fresh weight of each sample, and was somewhat higher when dry weight was used as the basis for comparison. Since it was impossible to obtain peeled mesophyll free of both epidermal layers, the values reported indicate only that the four samples do not differ greatly in their content of *o*-coumaric acid and the coumarin-glucosides.

Distribution of β-Glucosidase in the Leaves. The β-glucosidase was readily detected both in extracts from mesophyll tissue, which had the upper epidermis still attached, and in lower epidermis, which had been peeled from the leaf. Table II shows that the enzyme concentrations per mg of protein again were similar in the two samples. Since the mesophyll in a gram of intact leaves contains approximately 15 (19.3 ÷ 1.25) times as much protein, it follows that this tissue contained more than 90% of the β-glucosidase found in the leaf. This conclusion is also supported by the enzyme concentration observed in samples of isolated tissue, expressed on a fresh-weight basis.

Subcellular Localization of β-Glucosidase. When a homogenate of *M. alba* leaves was subjected to differential centrifugation, as described under "Materials and Methods," and the various fractions were examined, more than 85% of the β-glucosidase activity present initially in the homogenate was recovered in the 100,000*g* supernatant fraction. This fraction was devoid of Chl.

In an attempt to refine the system further, mesophyll protoplasts were prepared from young leaves and ruptured gently to obtain chloroplast preparations, which were more than 50% intact as judged by Chl and trioseP dehydrogenase distribution studies. Figure 1, which demonstrates the separation of intact chloroplasts from broken chloroplasts by sucrose density gradient centrifugation, also shows that the β-glucosidase activity did not move into the gradient during centrifugation. More than 95% of the activity applied to the gradient was recovered in the soluble fraction at the top of the gradient. It was also surprising to find that the specific activity of the β-glucosidase in the protoplast extracts (0.54 μmol/min·mg Chl) was less than 2% of that in whole leaf extracts (28.5 μmol/min·mg Chl).

Subcellular Localization of the Glucosides of o-Coumaric and Coumarinic Acids. Since the mesophyll tissue appears to contain not only the glucosides of *o*-coumaric and coumarinic acids but also the glucosidase which hydrolyzes coumarinyl glucoside, we obtained information on the localization of these compounds within the mesophyll cell. Boudet *et al.* (3) have developed a method for preparing vacuoles from mesophyll protoplasts of *M. alba*. Such vacuoles contain no more than 2% protoplasts as an impurity and are prepared in yields of approximately 30%, assuming that each protoplasts forms one vacuole on lysis.

Table III shows the analysis of three samples of protoplasts and vacuoles obtained from such protoplasts; these samples were prepared in France, lyophilized, and shipped to California for analysis. Samples I and II were prepared from the Spanish variety

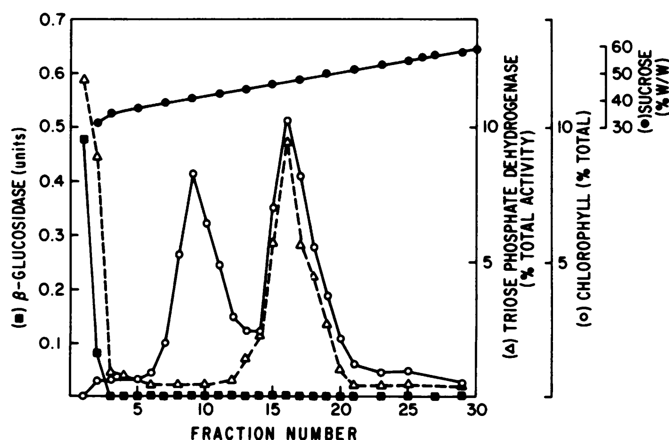

FIG. 1. Continuous sucrose gradient centrifugation of an extract of mesophyll protoplasts of *M. alba* centrifuged for 45 min at 25,000 rpm. Fractions (1.35 ml) were assayed for coumarinyl-β-glucosidase activity (■), Chl content (O), and NADPH trioseP dehydrogenase activity (Δ), and sucrose density (●). (See "Materials and Methods" for details).

Table III. *The Content of Free o-Coumaric Acid and Total 2-Hydroxycinnamic Acid in Vacuoles and Protoplasts from Mesophyll Tissue of M. alba Leaves*

Samples I and II were prepared from the Spanish variety of sweet clover. Sample III was prepared from the genotype CuCubb Nebraska code 745, seeds of which were kindly furnished by Dr. H. Gorz. The analyses for free and total coumaric acid were carried out as described in "Materials and Methods."

Samples	Total 2-Hydroxycinnamic Acid		Free *o*-Coumaric Acid	
	10^6 Vacuoles	10^6 Protoplasts	10^6 Vacuoles	10^6 Protoplasts
			nmol	
I	235	197	3.2	173
II	239	209	2.7	162
III	271	270	2.4	2.0

of *M. alba* used in all other work in this paper; sample III was prepared from the genotype CuCubb, Nebraska Code 745, which is low in β-glucosidase activity.

The data show that the total 2-hydroxycinnamic acid content of 10^6 vacuoles in all three samples is equal to (or even exceeds by 15% in sample I) the total 2-hydroxycinnamic acid content of 10^6 protoplasts. Thus, the vacuole appears to contain all of the 2-hydroxycinnamic acid compounds (free *o*-coumaric acid plus the two coumarin glucosides) contained in protoplasts prepared from mesophyll tissue. Examination of the values for free *o*-coumaric acid, however, shows an interesting difference between samples I and II and sample III. In sample III, the free *o*-coumaric acid (2.0 nmol) in 10^6 protoplasts is less than 1% of the total 2-hydroxycinnamic acid (270 nmol) observed after autoclaving; this means that most of the 2-hydroxycinnamic acid in protoplasts in this sample was present in the form of glucosides of *o*-coumaric and coumarinic acid. In samples I and II, however, only about 20% of the 2-hydroxycinnamic acid was present as the two glucosides. In the vacuoles of all three samples, free *o*-coumaric acid accounted for less than 2% of the total 2-hydroxy cinnamic acid detected after autoclaving.

Subcellular Localization of the Coumarin-β-Glucosidase and Glucosyl Transferase. When protoplasts and vacuoles isolated from mesophyll cells were examined for the β-glucosidase, the results given in Table IV were obtained. These samples were prepared in France and analyzed immediately. The vacuoles contained no detectable β-glucosidase activity, and protoplasts equivalent to 1 g of leaflets contained less than 1% (0.8%) of the enzyme present in the leaf extract. This finding, which was also indicated in the study reported in Figure 1, is not due to inactivation of the enzyme during preparation of the protoplasts or vacuoles, because all of the activity observed in the leaf extract was recovered in the digestion media (fractions III and IV, Table IV).

The localization of the glucosyl transferase, which catalyzes the glucosylation of *o*-coumaric acid (10), is also given in Table IV. Here, again, this enzyme was absent from the vacuole, but, in contrast to the β-glucosidase, 92% of the transferase present in the leaf extract was detected in the equivalent amount of protoplasts isolated from clover leaflets.

Studies with Cells Isolated from Mesophyll Tissue. Other laboratories (9, 17) have described β-glucosidases, possibly involved in lignification, which are firmly associated with the plant cell wall. Such enzymes are solubilized and, thereby, removed from the wall only after treatment with salt or buffer solutions of high ionic strength. This possibility was examined for the coumarinyl glucosidase by preparing individual cells or cell clusters which still possessed their cell walls. Analysis of such cells (fraction III, Table

Table IV. *Distribution of Coumarinyl β-Glucosidase and UDPG:o-Coumaric Acid Glucosyl Transferase in Protoplasts and Vacuoles from Sweet Clover Mesophyll Tissue*

Three different samples (1 g fresh weight each) of leaflets were removed from the same plant and extracted directly for enzyme analysis (I) or for the isolation of protoplasts and vacuoles. Enzyme analyses were subsequently carried out on extracts of the protoplasts (II) and vacuoles (V). The second digestion medium (Fraction IV) includes the two washing media resulting from protoplast purification.

Fraction	β-Glucosidase Activity		Glucosyl Transferase Activity	
	μmol/min·fraction	*% of fraction I*	*nmol/min·fraction*	*% of fraction I*
I. Leaflets (1 g)	74.7	100	18.5	
II. Protoplasts (from 1 g of leaflets)	0.2	0.3 (0.8)[a]	5.8 (17.1)[c]	92.4[c]
III. 1st digestion medium	29.5	39.5 (36.5)[b]	ND[d]	ND[d]
IV. 2nd digestion medium	51.0	68.3 (66.3)[b]	ND[d]	ND[d]
Total of fractions (II, III, IV)	80.7	108.1 (103.6)	17.1[c]	92.4[c]
V. Vacuoles (from 1 g of leaflets)	0	0	trace	trace

[a] Value corrected for protoplast yield: 38% of the cells in fraction I were recovered as protoplasts in fraction II, using Chl as a reference.

[b] Values corrected for the small amount of coumarinyl glucosidase activity in the preparation medium before digestion.

[c] Values corrected for a protoplast yield of 34% using Chl as a reference.

[d] ND, Activity not determined.

Table V. *Distribution of Coumarinyl Glucosidase Activity in Isolated Cells from Sweet Clover Mesophyll Tissue*

Two different samples (1 g fresh weight each) of leaflets were removed from the same plant and extracted directly for enzyme analysis (I) or used for cell isolation. Enzyme analyses were subsequently carried out on extracts of these isolated cells (II).

Fraction	β-Glucosidase Activity	
	μmol/min·fraction	*% of fraction I*
I. Leaflets (1 g)	71.3	100
II. Isolated cells (from 1 g of leaflets)	0.3	7.3[a]
III. Infiltration medium	14.3	20.1
IV. Digestion medium	51.7	72.5
Total of fractions (II, III, IV)	66.3	99.9

[a] Value corrected for cell yield: 13% of the cells in fraction I were recovered as intact cells in fraction II using Chl as a reference.

V) for β-glucosidase disclosed that only about 7% of the glucosidase present initially in a leaf homogenate was associated with such cells after correction for yield. Again, most of the activity was recovered in the medium used in preparing and purifying the isolated cells.

DISCUSSION

It is generally assumed that the glucosides of 2-hydroxycinnamic acid are physically separated within *M. alba* leaves from the hydrolyzing enzyme (a β-glucosidase), which catalyzes the hydrolysis of coumarinyl glucoside. In sorghum seedlings, Kojima *et al.* (11) have shown that dhurrin and its degradative enzymes (a dhurrin-specific β-glucosidase and a hydroxynitrile lyase) are located in the epidermis and mesophyll tissues, respectively. This compartmentation at the tissue level does not occur in the case of *M. alba*, however, since Tables I and II demonstrate that the glucosidase and the coumarin-glucosides exist in both tissues.

Differential centrifugation, as well as the data reported in Figure 1, shows that the coumarin-β-glucosidase in sweet clover leaves is soluble and not associated with any Chl-containing fractions (*e.g.* intact chloroplasts or chloroplast fragments). This contrasts with the observations of Thayer and Conn (21), who found the dhurrin-specific β-glucosidase of *Sorghum bicolor* to be associated with mesophyll chloroplasts.

The availability of a procedure (3) for isolating vacuoles from mesophyll protoplasts of *M. alba* made it possible to examine the vacuolar distribution of these glucosides and the related glucosidase. The data in Table III show that the vacuoles of mesophyll cells contain all of the 2-hydroxycinnamic acid compounds (free *o*-coumaric acid and related glucosides) found in the mesophyll protoplasts. The fact that the vacuolar content actually exceeds, by 15% in one sample, that of the protoplasts may be due in part to lysis of some vacuoles during collection and before counting. The discrepancy could not be attributed to Ficoll, mannitol, or dextran sulfate used in isolation of the vacuoles, since these compounds did not affect the fluorometric analysis. Similar discrepancies, *i.e.* excess of vacuole over protoplast contents, have been observed by Saunders (19) and by Boller and Kende (4).

Vacuoles isolated from mesophyll protoplasts of *M. alba* were devoid of β-glucosidase capable of hydrolyzing the coumarinyl glucoside found in the vacuoles (Table IV). This compartmentation of substrate and related enzyme at the cellular level explains the production of free coumarin, which occurs only after the cellular structure of the clover leaf is disrupted.

The low recovery (less than 1%, Table IV) of β-glucosidase in protoplast preparations, compared with the equivalent amount of leaf homogenate, clearly suggests that most of this enzyme is located in the apoplast. Other workers (17) have obtained similar data and have interpreted such results to indicate that the β-glucosidase is associated with the cell wall. Still other workers (9, 16) have reported that cell wall fractions contain β-glucosidases active on secondary plant products. When isolated cells of *M. alba* mesophyll were prepared and examined for the β-glucosidase, only a small fraction (7%) of the total activity was observed. These

results suggest either that this β-glucosidase is loosely attached to the cell wall and easily removed during preparation of the isolated cells or that the enzyme is located primarily in the intercellular spaces of the intact leaf.

The recovery of essentially all of the *o*-coumaric acid:UDPG glucosyl transferase of a leaf homogenate in an equivalent amount of protoplasts demonstrates that this enzyme is located within the mesophyll cell, in contrast to the β-glucosidase. Since vacuoles prepared from these protoplasts lacked the transferase, this enzyme is located exterior to the vacuole. Such location could be consistent with a role for this enzyme in glucosylating the phenolic *o*-coumaric acid prior to its transfer into and storage in the vacuole.

The data in Table III also showed that most of the 2-hydroxycinnamic acid present in protoplasts occurred as glucosides in sample III, whereas only 20% was present as glucosides in samples I and II. Since most of the 2-hydroxy-*trans*-cinnamic acid in commercial varieties of *M. alba* (such as Spanish) is present as glucosides, these compounds must have been extensively hydrolyzed in samples I and II during the period (approximately 14 days) between isolation of the protoplasts and vacuoles in Toulouse, France, and their lyophylization, shipment, and analysis in Davis, CA. (Similarly, the relatively large amount of free *o*-coumaric acid in the samples described in Table I probably is due to handling of the leaves in that study.) This occurred even though the β-glucosidase present in the protoplasts of these samples was only a small fraction of the enzyme present in an equivalent amount of intact leaf. The large extent of hydrolysis observed also means that much of the *o*-coumaric glucoside present initially was isomerized, possibly by light (6), to form the coumarinyl glucoside which was hydrolyzed. On the other hand, protoplasts prepared from plants genetically deficient in β-glucosidase (sample III) contained very little free *o*-coumaric acid. The fact that the free *o*-coumaric acid content of the vacuoles in all three samples is less than 1% of the total 2-hydroxy cinnamic acid in the vacuoles supports this explanation and is consistent with the observation (Table IV) that the vacuoles of mesophyll cells do not contain any of the β-glucosidase.

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