Isoflavone and Pterocarpan Malonylglucosides and β-1,3-Glucan- and Chitin-Hydrolases are Vacuolar Constituents in Chickpea (*Cicer arietinum* L.)

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Cell suspension cultures of chickpea (*Cicer arietinum* L.) were used to prepare protoplasts and vacuoles. The vacuolar preparation revealed only slight contaminations of cytoplasmic marker enzymes. HPLC analysis of the vacuolar extract showed that the malonylglucosides of isoflavones, isoflavanones and pterocarpans are exclusively located in the vacuole. Experiments designed to determine the subcellular localization of the isoflavone malonylglucoside: malonylesterase suggest an association of this enzyme with the vacuolar membrane. Finally, a β-1,3-glucanase and a chitinase with basic isoelectric points were also found to be localized in the chickpea vacuoles.

Introduction

Vacuoles play an important role for the storage of secondary metabolites in plants [1–4]. This compartmentation ensures the efficiency of their production and avoids harmful effects on the cells. Synthesis, degradation and storage of secondary constituents is controlled by the permeability properties of membranes. The control of this transport and the vacuolar storage play a key role in achieving high production of these compounds. In addition to numerous cases listed by Guern *et al.* [3] it is to be expected that further hydrophilic secondary compounds will be determined as vacuolar constituents. Besides flavonoids, anthocyanins, isoflavonoids and cardiac glycosides a great number of alkaloids are also known to be stored in plant vacuoles. Guern *et al.* [3] and Renaudin and Guern [5] have discussed in great detail the inherent mechanisms of tonoplast transport and storage of the compounds investigated so far.

Chickpea cell suspension cultures constitutively accumulate the isoflavones biochanin A, formononetin, homoferreirin and cicerin as major phenolics. These compounds predominantly occur in form of malonylglucoside conjugates (Fig. 1). The enzymes catalyzing the four reactions involved in the metabolism of the isoflavone and pterocarpan conjugates, an isoflavone 7-O-glucoside-glucosyltransferase [6], an isoflavone 7-O-glucoside-6″-O-malonyltransferase [7], an isoflavone 7-O-glucoside-6″-O-malonylesterase [8] and specific β-glucosidases [9] have been studied. Numerous investigations have amply demonstrated the increasing importance of malonyl conjugates in plant metabolism. Several classes of secondary constituents, D-configurated amino acids, endproducts of pesticide degradation and intermediates of phytohormon production were described as malonyl conjugates [10, 11]. In addition to chickpea and other previously analyzed plants [12] very recent investigations also demonstrated that the isoflavones in soybean and alfalfa mainly accumulate as 7-O-glucoside-6″-O-malonates [13, 14]. Furthermore, the pterocarpan phytoalexins of chickpea cell suspension cultures have been found to accumulate as malonyl conjugates [15]. For apigenin-malonylglucoside in parsley [16] and the acylated anthocyanin of *Daucus carota* [17] a vacuolar localization has been shown earlier. A vacuolar localization of the isoflavone and pterocar-

Abbreviations: B, biochanin A; BG, biochanin A 7-O-glucoside; BGM, biochanin A 7-O-glucoside-6″-O-malonate; F, formononetin; FG, formononetin 7-O-glucoside; FGM, formononetin 7-O-glucoside-6″-O-malonate; Me; medicarpin; Ma, maackiain;MeGM, medicarpin 3-O-glucoside-6′-O-malonate; MaGM, maackiain 3-O-glucoside-6′-O-malonate; CGM; cicerin 7-O-glucoside-6″-O-malonate; HGM, homoferreirin 7-O-glucoside-6″-O-malonate; AUFS, absorption full scale; AC, acidic chitinase; BC, basic chitinase; BG, basic glucanase; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; pI, isoelectric point; CM-Chitin-RBV, carboxymethylated chitin-remazol brilliant violet.

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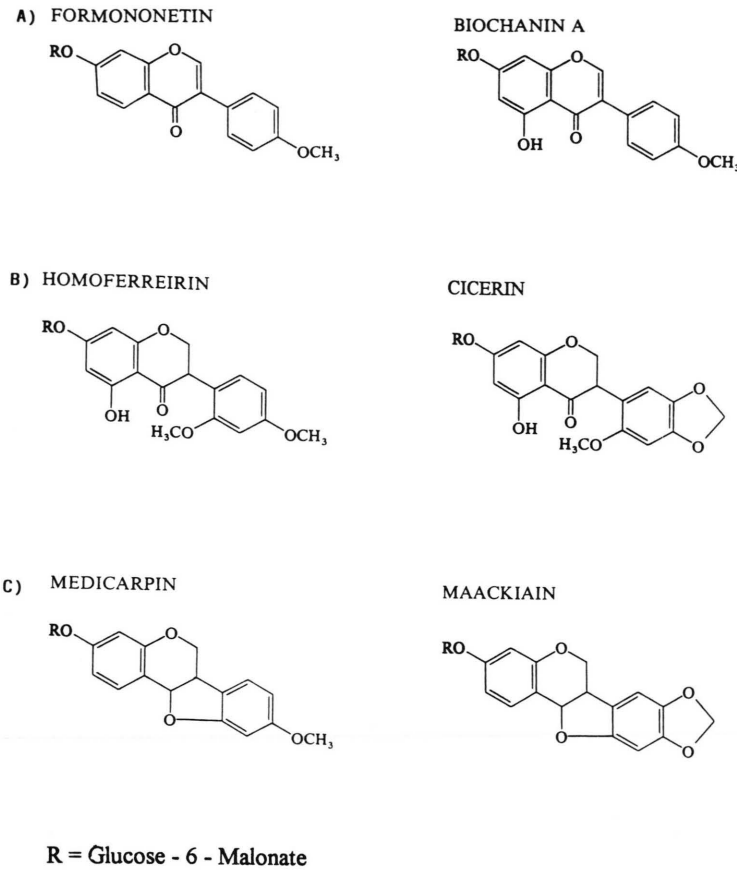

Fig. 1. Structures of A) isoflavones, B) isoflavanones, and C) pterocarpans occuring as malonylglucosides in chickpea.

pan malonylglucosides of chickpea has not been proven clearly until yet.

Apart from the accumulation of phytoalexins the synthesis of pathogenesis-related proteins is an especially prominent reaction of plants after microbial infection or elicitation [18]. Recent investigations have identified different functions for pathogenesis-related proteins as β-1,3-glucanases and chitinases [19, 20]. These enzymes are thought to play an important role in the plant defence system due to their ability to inhibit fungal growth by degrading components of the mycelial cell wall. Hydrolases with a basic pI were found to be predominantly located in the vacuole, whereas acidic hydrolases were secreted into the extracellular compartment [21, 22]. From chickpea cell cultures two chitinases with an acidic and a basic pI respectively, and one basic β-1,3-glucanase have been purified ([23], Vogelsang and Barz, submitted).

We have now isolated protoplasts and vacuoles from chickpea cell suspension cultures to analyze and compare the pattern of phenolic constituents and the cellular distribution of the activities of the two chitinases and the one β-1,3-glucanase. The results of the investigations presented in this paper again show that the vacuole is an important storage site.

Materials and Methods

Cell cultures

Chickpeak cell suspension cultures were grown as described previously [24].

Protoplast isolation

Protoplasts were isolated from 3–5 days old cell suspension cultures. Cells (5–7 g fresh weight) were incubated in 50 ml filter-sterilized maceration medium composed of 2% Onozuka R 10 cellulase (Yakult, Honsha, Japan), 0.3% macerocyme R-10 (Serva, Heidelberg, F.R.G.), 0.25% hemicellulase (Sigma, Munich, F.R.G.) in modified PRL-4c me-

dium [24, 25] containing 0.25 M mannit and 0.25 M sorbit. The cells were incubated for 16 h at 24 °C and agitated with 90 rpm in this maceration medium. After incubation the protoplast suspension was filtered through two layers of nylon cloth (mesh size 150 µm and 60 µm, respectively). The protoplasts were pelleted by centrifugation (5 min, 200 × *g*) in conical reaction tubes. The supernatant was discarded and the pellet was washed twice with the above described medium lacking the enzymes. The yield of protoplasts was determined by counting an aliquot in a Fuchs-Rosenthal chamber under a microscope. The digestion of the cell walls during liberation of the protoplasts and the viability of the protoplasts could be observed under a fluorescence microscope after staining with calcofluor white and fluoresceine diacetate.

Vacuole isolation

Vacuoles were released from freshly isolated protoplasts (*ca.* 5×10^6 protoplasts) by addition of 5 ml lysis medium containing 10% Ficoll 400 (Pharmacia, Freiburg, F.R.G.), 20 mM EDTA, 2 mM DTT and 0.2 M mannit in 5 mM Hepes-KOH buffer, pH 8. The protoplasts were incubated with the lysis medium for 5 min at 40 °C [26]. After lysis the suspension was overlayered with 2 ml 4% Ficoll 400 (in 1 vol. of the above mentioned buffer and 1.5 vol. 10 mM Hepes-KOH buffer, pH 7.5 containing 0.5 M mannit) and 1 ml 10 mM Hepes-KOH buffer, pH 7.5, containing 0.5 M mannit and centrifuged (2000 × *g*) at 4 °C for 25 min. After centrifugation the vacuoles remained at the interface of the buffer and the 4% Ficoll. The yield of vacuoles was determined by counting an aliquot in a Fuchs-Rosenthal chamber under a microscope.

Enzyme assays

NADPH:cytochrome *c* reductase was assayed according to Bergmeyer [27]. Glucose-6-phosphate-dehydrogenase and NADH-malate-dehydrogenase were assayed spectrophotometrically by monitoring changes in absorption at 340 nm according to Bergmeyer [27].

α-Mannosidase and acid phosphatase were assayed by measuring the release of *p*-nitrophenol, using substrates and procedures described by Boller and Kende [28]. Mg^{2+}/K^+-ATPase was determined following the protocol of Hodges and Leonard [29].

Isoflavone 7-O-glucoside-glucosidase, isoflavone 7-O-glucoside-6″-O-malonyltransferase, isoflavone 7-O-glucosyltransferase and isoflavone malonylglucoside:malonylesterase were measured according to our published procedures [6–9].

β-1,3-Glucanase activity was measured as described by Vogelsang and Barz [23].

Chitinase activity was determined according to Wirth and Wolf [30]. Aliquots of enzyme solutions (1–10 µl) were incubated in 600 µl 0.1 M potassium acetate buffer, pH 5.0 at 37 °C. Enzyme reaction was started by the addition of 200 µl CM-Chitin-RBV (Blue Substrates, Göttingen, F.R.G.). Addition of 200 µl 1 N HCl after 5 min stopped the reaction and caused precipitation of the unhydrolyzed substrate. After cooling on ice and centrifugation (1 min, 3000 × *g*) the released and soluble CM-Chitin-RBV fragments were obtained in the supernatant. The increase of optical density at 550 nm against a blank assay prepared without enzyme solution was measured photometrically (PM 6, Zeiss, Oberkochen, F.R.G.) at 550 nm (Δ OD_{550}). The release of soluble CM-Chitin-RBV fragments was a linear function of enzyme activity up to a Δ OD_{560} of 0.35. Therefore, a dilution series of the enzyme solution was prepared to reach this linear range. One unit was defined as $\Delta\, OD_{560} \cdot ml^{-1} \cdot min^{-1}$.

The protein content of the enzyme extracts was determined by the method of Bradford [31] using bovine serum albumin as a standard.

Isolation of phenolic constituents from protoplasts and vacuoles of chickpea

Freshly isolated protoplasts ($2-4 \times 10^6$) were extracted with 10 ml acetone/methanol (1:1). The extracts were brought to dryness and the residue was dissolved in 1 ml methanol. The isolated vacuoles ($1-1.5 \times 10^6$) were acidified with 1 N HCl to pH 2 and extracted twice with 10 ml ethylacetate. The ethylacetate fractions were brought to dryness and the residue was dissolved in 200 µl methanol. These extracts were used for analysis and quantification of the phenolic compounds by HPLC-techniques as described earlier [12, 32].

Preparation of tonoplast fragments

After freezing and thawing of the vacuolar preparation the tonoplast fragments were obtained by

lysing the vacuoles (appr. 2×10^6 organells) in 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA [33]. The mixture was subsequently centrifuged (100000 × *g*, 1 h) [34]. The pellet was resuspended in buffer and the solution was used as a tonoplast fraction for the enzyme assays.

Anion-exchange chromatography

In order to determine the subcellular localization of the chitinases and the β-1,3-glucanase, extracts from vacuoles, protoplasts, cell culture cells and medium each containing 0.5 mg protein were loaded on a FPLC Mono P HR 5/5 column with a high pH of 10.7 to allow binding of the hydrolases with a basic pI. Hydrolases were eluted by an increasing NaCl-gradient from 0 to 0.2 M. Fractions (0.5 ml) were assayed for individual specific β-1,3-glucanase and chitinase activity. Activities of the basic β-1,3-glucanase (BG) and the basic chitinase (BC) were compared with the specific activity of the extracellularly accumulating acidic chitinase (AC) as a marker enzyme during each run.

Results and Discussion

Protoplast and vacuole isolation

Cell cultures of chickpea turned out to be an excellent system to prepare protoplasts that can be further utilized to isolate vacuoles and to analyze the content of secondary metabolites. Using the protocol described under Materials and Methods about 30–40% of the cells were liberated as protoplasts. 80–90% of the protoplasts exhibited a good viability after incubation with fluoresceine diacetate. Residual cell wall fragments could not be detected after calcofluor white staining.

For vacuole isolation the protoplasts were routinely used directly after isolation. Satisfactory lysis of the protoplasts was obtained by incubation in 5 mM Hepes-KOH buffer containing 0.2 M mannit and 10% Ficoll 400 [26]. This method led to rupture of nearly 80% of the protoplasts. For purification the vacuoles were centrifuged through a Ficoll-gradient. The yield of vacuoles after this purification step varied from 20–30% depending on the initial number of protoplasts. Further purification was leading to less cytoplasmatic contamination, but resulted in a decrease of vacuole yield. Other methods like liberation of the vacuoles by ultracentrifugation techniques [35], incubation with the polycation DEAE-dextran [36, 37] or osmotic shock [38, 39] were also tested but did not lead to satisfactory results. Therefore, our established vacuole isolation protocol seemed to be useful for large scale isolation of an intact vacuolar fraction in a rather short time. For further experiments the vacuoles were kept at 4 °C. Under these conditions the vacuole preparation was stable for some hours.

Vacuole characterization

The quality of the isolated vacuoles was determined by microscopic techniques and by assaying marker enzymes. Microscopic examination of the vacuole preparation revealed no contamination with intact protoplasts or protoplast fragments.

In order to test the purity of the vacuolar fraction activities of some marker enzyme were measured both in the protein extracts of protoplasts and vacuoles. The distribution of marker enzymes in the protoplast and in the vacuolar preparation is shown in Table I. Each value represents the

Table I. Enzyme activities and percent distribution in protoplasts and vacuoles isolated from chickpea cell suspension cultures. Total enzyme activities (nkat) per 10^6 protoplasts or vacuoles are means of at least 3 separate experiments. Deviations were not higher than 10%.

Enzyme	Protoplasts	Vacuoles
Glucose-6-phosphate dehydrogenase	1.6 (100%)	no activity (0%)
Malate-dehydrogenase	12.5 (100%)	1.7 (13%)
Cytochrome *c*-reductase	0.14 (100%)	0.013 (10%)
α-Mannosidase	0.9 (100%)	1.1 (120%)
Acid phosphatase	1.7 (100%)	2.0 (117%)
K^+/Mg^{2+}-ATPase	1.43 (100%)	1.19 (83%)
Isoflavone glucoside glucosidase	0.072 (100%)	0.008 (13%)
Isoflavone glucoside malonyltransferase	0.06 (100%)	0.003 (5%)
Isoflavone malonylglucoside malonylesterase	0.595 (100%)	0.508 (85%)
Isoflavone glucosyltransferase	0.00067 (100%)	0.00003 (5%)

means of at least three replicate experiments and is calculated on the basis of specific activity and on the basis of the number of isolated protoplasts and vacuoles. Glucose-6-phosphate-dehydrogenase and malate-dehydrogenase, two enzymes which are believed to be exclusively located in the cytosol, were only detected in very small amounts in the vacuolar fraction. NADPH cytochrome *c*-reductase was used as marker enzyme for the endoplasmatic reticulum. Compared with the protoplasts the vacuoles contained approximately 15% of this enzyme. These data are comparable with results from other publications [38–40]. As marker for the vacuolar compartment several hydrolytic enzymes were determined. α-Mannosidase, acid phosphatase and Mg^{2+}/K^{+}-ATPase are known to be localized in the vacuoles [28, 40–42]. The chickpea vacuoles also contained high levels of α-mannosidase, acid phosphatase and ATPase activity (Table I). These data indicate a highly purified vacuolar fraction, showing only a slight contamination with other cell organelles. Determination of the protein content showed that 15–20% of the protoplast protein could be ascribed to the vacuoles. These results are in agreement with literature data on other plant vacuoles [43].

Apart from these marker enzymes the intracellular distribution of chickpea enzymes involved in isoflavone and pterocarpan conjugation metabolism were analyzed. Isoflavone 7-O-glucoside-glucosidase, isoflavone 7-O-glucoside-6″-O-malonyltransferase and isoflavone 7-O-glucosyltransferase seem to be cytoplasmatic enzymes, because only low activities (up to 15%) of these enzymes were found in isolated chickpea vacuoles. In contrast, the isoflavone-malonylesterase appears to be localized in the vacuole. 85% of this enzyme activity was measured in the vacuolar fraction (Table I).

Accumulation of β-1,3-glucan- and chitin-hydrolases in chickpea vacuoles

Furthermore, the subcellular localization of β-1,3-glucanase and chitinase was investigated. These enzymes belong to the pathogenesis-related proteins and have been implicated in defence reactions of plants against potential pathogens [44]. Enzyme extracts were prepared from vacuoles, protoplasts, cells and medium and 0.5 mg protein of each extract was separated by anion-exchange chromatography on FPLC Mono P. The basic β-1,3-glucanase (BG) and the basic chitinase (BC) exhibited their highest specific enzyme activities in extracts from vacuoles and protoplasts (Fig. 2A, B). In contrast, the specific activity of the acidic chitinase (AC) was low in these extracts, but

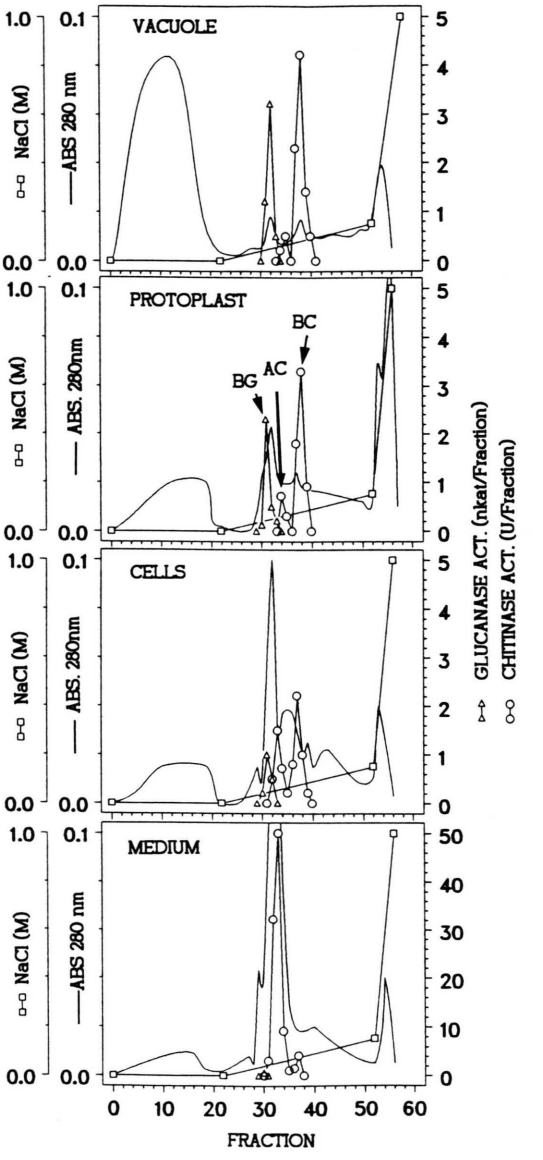

Fig. 2. FPLC-chromatograms of protein extracts from 4 different compartments. A, vacuole; B, protoplast; C, cells; D, medium. From each compartment an identical amount of protein (0.5 mg) was fractionated on FPLC Mono P. Eluted fractions (0.5 ml) were assayed for β-1,3-glucanase (△–△) and chitinase (○–○) activity. The sequence of elution of the 3 chickpea hydrolases (BG, AC, BC) is indicated in Fig. 2B.

increased in cells and especially in the medium in relation to the basic hydrolases (Fig. 2C, D). Thus, the chromatography patterns strongly suggest a vacuolar localization of the basic β-1,3-glucanase and basic chitinase and a secretion of the acidic chitinase into the extracellular space. This finding is in accordance with localization studies in bean and tobacco [21, 22], where a vacuolar localization of basic hydrolases has been reported.

Phenolic constituents of chickpea vacuoles

The isoflavones and pterocarpans of chickpea cell suspension cultures predominately accumulate as 7-O-glucoside-6″-O-malonates or 3-O-glucoside-6-O-malonates, respectively (Fig. 1). To determine the subcellular localization of these compounds methanolic extracts of chickpea protoplasts and vacuoles were analyzed by HPLC [12, 32]. The HPLC chromatograms of both extracts are shown in Fig. 3. The extract of the protoplasts fraction revealed a similar pattern of secondary compounds as found in extracts prepared from cells (data not shown). The treatment of cells with digestive enzymes for the preparation of protoplasts did not lead to any liberation of the malonylglucosides or to a significant induction of phytoalexin accumulation. Protoplast and vacuole extracts share a similar pattern of secondary compounds (Fig. 3A, 3B) indicating a common storage site for the malonylconjugates BGM, FGM, HGM, CGM [12, 32] and the malonylglucosides of the phytoalexins medicarpin and maackiain [15]. Only low amounts of the isoflavone-glucosides BG and FG as well as their respective aglyca could be detected in the vacuolar extracts.

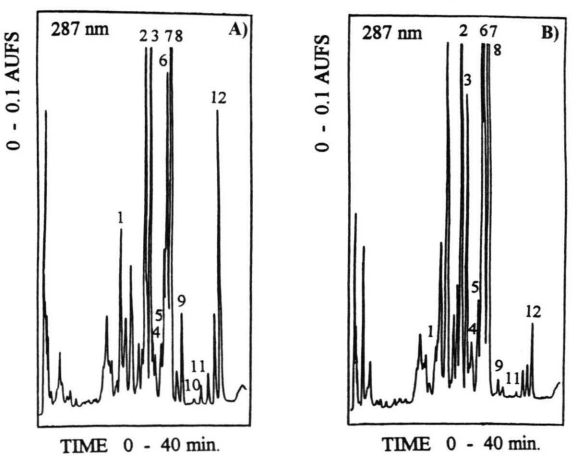

Fig. 3. HPLC-chromatogram of a methanolic extract from chickpea protoplasts (A) and chickpea vacuoles (B). The compounds are: 1: FG, 2: FGM, 3: BG, 4: MaGM, 5: MeGM, 6: CGM, 7: BGM, 8: HGM, 9: F, 10: Ma, 11: Me, 12: B.

Table II shows the amounts of the major isoflavone and pterocarpan compounds in protoplasts and vacuoles. Comparing both compartments on a basis of 10^6 protoplasts or vacuoles, the vacuoles contained essentially identical amounts of the malonylconjugates but by far lower amounts of the corresponding glucosides or aglyca. These results suggest that the aglyca and the glucosides did not accumulate constitutively in the vacuoles but rather originate from the malonylconjugates by the action of the degradative enzymes isoflavone-glucosidase and isoflavone-malonylesterase during organell preparation. Furthermore the data clearly corroborate that the conjugates BGM, FGM, CGM, HGM, MeGM, and MaGM are stored in vacuoles and therefore these compounds have to be added to the list of documented vacuolar secondary constituents [3–5].

The high level of the isoflavone-malonylglucoside : malonylesterase activity detected in the vacuolar fraction (Table I) raised the question of the subcellular localization of this enzyme. A strict compartimentation of synthesis, storage and degradation of secondary compounds is one important prerequisite for an intact plant metabolism. Therefore it might not be very favourable that the

Table II. Amounts of isoflavones, isoflavanones, pterocarpans and their conjugates (nmol/10^6) and percent distribution in protoplasts and vacuoles isolated from chickpea cell suspension cultures. For number of compounds see Fig. 3. The data are means of at least 3 separate experiments.

Compound		Protoplasts		Vacuoles	
Biochanin A	(12)	23	(100%)	6	(25%)
BG	(3)	58	(100%)	21	(36%)
BGM	(7)	132	(100%)	128	(97%)
Formononetin	(8)	8	(100%)	2	(25%)
FG	(1)	14	(100%)	4	(28%)
FGM	(2)	38	(100%)	39	(102%)
CGM	(6)	103	(100%)	115	(110%)
HGM	(9)	45	(100%)	41	(93%)
Medicarpin	(11)	5	(100%)	1	(20%)
Maackiain	(10)	1	(100%)	not found	
MeGM	(5)	16	(100%)	15	(94%)
MaGM	(4)	16	(100%)	17	(105%)

substrates (malonylglucosides) and the degradative enzyme (isoflavone-malonylglucoside: malonylesterase) both occur in the vacuole. Previous investigations by Hinderer *et al.* [45] suggested a membrane association of this enzyme. Therefore to elucidate the question of subcellular distribution we have isolated tonoplast fragments by ultracentrifugation [33, 34] and measured the activity of the malonylesterase both in the tonoplast fragments and in the vacuolar supernatant. The results are listed in Table III. The vacuolar fraction exhibited an enzyme activity of 0.58 nkat/10^6 vacuoles. After ultracentrifugation nearly 62% of the malonylesterase activity and 45% of the tonoplast associated K^+/Mg^{2+}-ATPase were measurable in the tonoplast fragments. The vacuolar supernatant also contained more than 90% of the activities determined for peroxidase, acid phosphatase and α-mannosidase. This distribution of the analyzed enzymes strongly points to an association of the malonylesterase with the tonoplast membrane.

Table III. Absolute (nkat) and relative (%) distribution of enzyme activities between vacuolar content and tonoplast membrane based on 10^6 vacuoles.

Enzymes	Vacuole	Tonoplast	Vacuolar content
Isoflavone malonylglucoside: malonylesterase	0.508 (100%)	0.315 (62%)	0.150 (30%)
K^+/Mg^{2+}-ATPase	1.19 (100%)	0.54 (45%)	0.38 (32%)
Peroxidase	22 (100%)	0.9 (4%)	24 (109%)
Acid phosphatase	2.0 (100%)	0.2 (10%)	1.9 (95%)
α-Mannosidase	1.1 (100%)	0.08 (7%)	1.0 (91%)

In chickpea the malonylglucoside of the isoflavone formononetin and the malonylconjugates of the two pterocarpan phytoalexins do not accumulate irreversibly in the vacuole [46]. Upon elicitation the isoflavone formononetin can be liberated from the glucoside and after excretion from the vacuole it is funneled into the phytoalexin biosynthesis. Furthermore the phytoalexin-malonylconjugates are also metabolized after elicitation and then accumulate as aglyca in the cells (Mackenbrock *et al.*, in prep.). Thus, the possible association of the malonylesterase with the tonoplast could represent an important regulatory system for the elicitor-induced cleavage of malonylconjugates. These rapidly available isoflavones and pterocarpans may then be used to defend the cell against an attacking pathogen.

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[1] F. Marty, D. Branton, and R. A. Leigh, in: The Biochemistry of Plants, Vol. **I,** The Plant Cell (N. E. Tolbert, ed.), p. 625, Academic Press, New York, London 1980.
[2] P. Matile, Naturwissenschaften **71,** 18–24 (1984).
[3] J. Guern, J. P. Renaudin, and S. C. Brown, in: Cell Culture and Somatic Genetics of Plants, Vol. **4,** Cell Culture in Phytochemistry (F. Constable and J. K. Vasil, eds.), p. 43, Academic Press, San Diego 1987.
[4] W. Kreis and H. Hölz, Naturwissenschaftl. Rundschau **44** (12), 463–470 (1991).
[5] J. P. Renaudin and J. Guern, in: Proceedings of the Phytochemical Society of Europe 30 (B. V. Charlwood and M. J. C. Rhodes, eds.), Oxford Science Publications 1990.
[6] J. Köster and W. Barz, Arch. Biochem. Biophys. **212** (1), 98–104 (1981).
[7] J. Köster, R. Bussmann, and W. Barz, Arch. Biochem. Biophys. **234** (2), 513–521 (1984).
[8] W. Hinderer, J. Köster, and W. Barz, Arch. Biochem. Biophys. **248** (2), 560–578 (1986).
[9] W. Hösel and W. Barz, Eur. J. Biochem. **57,** 607–616 (1975).
[10] W. Barz and J. Köster, in: The Biochemistry of Plants, Vol. **7,** Secondary Plant Products (E. E. Conn, ed.), p. 35, Academic Press, New York 1981.
[11] W. Barz, J. Köster, K. Weltring, and D. Stark, in: Proceedings of the Phytochemical Society of Europe 25, p. 307–347, Oxford Science Publication 1985.
[12] J. Köster, D. Strack, and W. Barz, Planta med. **48,** 131–135 (1983).
[13] T. J. Graham, J. E. Kim, and M. Y. Graham, Mol. Plant Microbe Interactions **3** (3), 157–166 (1990).
[14] H. Keßmann, R. Edwards, P. W. Gena, and R. A. Dixon, Plant Physiol. **94,** 227–232 (1990).
[15] C. Weidemann, R. Tenhaken, U. Höhl, and W. Barz, Plant Cell Rep. **10,** 371–374 (1991).
[16] U. Matern, W. Heller, and K. Himmelspach, Eur. J. Biochem. **133,** 439–448.
[17] W. Hopp and H. U. Seitz, Planta **170,** 75–85 (1987).
[18] L. C. Van Loon, Plant Mol. Biol. **4,** 111–116 (1985).
[19] S. Kauffmann, M. Legrand, P. Geoffroy, and B. Fritig, EMBO J. **6,** 3209–3212 (1987).
[20] E. Kombrink, M. Schröder, and K. Hahlbrock, Proc. Natl. Acad. Sci. U.S.A. **85,** 782–786 (1988).
[21] F. Mauch and L. A. Staehelin, The Plant Cell **1,** 447–457 (1989).
[22] M. van den Bulcke, G. Bauw, C. Castresana, M. van Montagu, and J. Vanderkerckhove, Proc. Natl. Acad. Sci. U.S.A. **86,** 2673–2677 (1989).
[23] R. Vogelsang and W. Barz, Z. Naturforsch. **45c,** 469–476 (1990).
[24] H. Keßmann and W. Barz, Plant Cell Rep. **6,** 55–59 (1987).
[25] O. L. Gamborg, Can. J. Bot. **44,** 791 (1966).
[26] M. T. Hauser and M. Wink, Z. Naturforsch. **45c,** 949–957 (1990).
[27] H. U. Bergmeyer, in: Methods of Enzymatic Analysis, Vol. **I,** Academic Press, New York 1974.
[28] T. Boller and H. Kende, Plant Physiol. **63,** 1123 (1979).
[29] T. Hodges and R. T. Leonard, Method. Enzymol. **32,** 392–406 (1974).
[30] S. J. Wirth and G. A. Wolf, J. Microbiol. Meth. **12,** 197–205 (1990).
[31] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[32] J. Köster, A. Zuzok, and W. Barz, J. Chromat. **270,** 383–395 (1983).
[33] F. Marty and D. Branton, J. Cell Biol. **87,** 72 (1980).
[34] E. Martinoia, E. Vogt, D. Rentsch, and N. Amrhein, Biochim. Biophys. Acta **1062,** 271–278 (1991).
[35] M. Thom, A. Maretzki, and E. Komor, Plant Physiol. **69,** 1315–1319 (1982).
[36] A. M. Boudet, H. Canut, and G. Alibert, Plant Physiol. **68,** 1354 (1981).
[37] K. Glund, A. Tewes, S. Abel, V. Lunhos, R. Walther, and H. Reinbothe, Z. Pflanzenphysiol. **113,** 151–161 (1983).
[38] F. Sasse, D. Backs-Hüsemann, and W. Barz, Z. Naturforsch. **34c,** 848–853 (1979).
[39]
[40] C. Buser-Suter, A. Wiemken, and P. Matile, Plant Physiol. **69,** 456–459 (1982).
[41] A. Admon, B. Jakoby, and E. Goldschmidt, Plant Sci. Lett. **22,** 89–96 (1981).
[42] K. Aoki and K. Nishida, Plant Physiol. **74,** 21–25 (1984).
[43] J. A. Saunders, Plant Physiol. **64,** 74–78 (1979).
[44] T. Boller, A. Gehri, F. Mauch, and U. Vögeli, Planta **157,** 22–31 (1983).
[45] W. Hinderer, J. Köster, and W. Barz, Z. Naturforsch. **42c,** 251–257 (1987).
[46] U. Mackenbrock and W. Barz, Z. Naturforsch. **46c,** 43–50 (1990).