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# Reversibility of the oleanolic acid monoglycosides transport across the tonoplast in vacuoles isolated from *Calendula officinalis* leaves*

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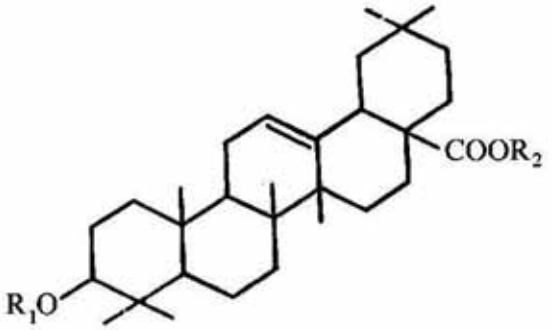
**The possibility of the reversible tonoplast transport of oleanolic acid monoglycosides was investigated in vacuoles isolated from *Calendula officinalis* leaf protoplasts. The obtained results point to the reversibility of the transport of monoglucoside I, whereas monoglucuronide F seems to be definitely stored in the vacuolar space.**

The vacuole is the largest compartment of a mature plant cell and serves as an internal reservoir of metabolites and nutrients. Vacuolar constituents vary between and within plant species, depending on the environmental conditions; this suggests that the transport of metabolites and nutrients across the vacuolar membrane, the tonoplast, is strictly controlled to permit optimal functioning of the cytoplasm. Research on vacuolar transport and its regulation may, therefore, be considered as an approach towards understanding of metabolic regulation of the plant cell. Thus, in the last years the transport of various compounds across the tonoplast has been intensively investigated; for example, we have demonstrated the transport of pentacyclic triterpenic acid monoglycosides (Fig. 1) into vacuoles isolated from marigold *(Calendula officinalis)* leaf protoplasts [1, 2].

*Calendula officinalis* leaves contain two series of oleanolic acid glycosides [3, 4], i.e. glucuronides (derivatives of 3-*O*-monoglucuronide) and glucosides (derivatives of 3-*O*-monoglucoside). Our previous studies have shown that, after their synthesis in the cytoplasm, almost 40% of oleanolic acid glycosides is transported across the tonoplast and




| | |
|---|---|
| Oleanolic acid | $R_1 = R_2 = H$ |
| 3-O-monoglucoside I | $R_1$ = Glc, $R_2 = H$ |
| 3-O-monoglucuronide F | $R_1$ = GlcUA, $R_2 = H$ |

**Figure 1. The structure of oleanolic acid and its monoglycosides present in *Calendula officinalis* leaves.**

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accumulated in the vacuole [5]. Detailed research on the process of this transport has evidenced that the two series of oleanolic acid glycosides differ in the mechanism of their transport to vacuoles. The transport of monoglucoside I and its derivatives is carrier-mediated and energy-dependent, whereas the transport of monoglucuronide F and other glucuronides is also a carrier-mediated, but a passive process (Fig. 2) [1, 2].

The role of the vacuole as the storage compartment is now well documented for many various compounds. Primary metabolites such as carbohydrates, amino acids and organic acids, as well as inorganic ions, are stored only temporarily in the vacuole and can be transported to the cytoplasm when needed. In contrast, many compounds of secondary plant metabolism (including triterpenoids) are regarded to be sequestered definitively within the vacuole [6]. However, there are some data suggesting the reversibility of the tonoplast transport of several alkaloids [7, 8]. The aim of our present studies was to examine whether the transport of oleanolic acid monoglycosides across the tonoplast could be reversible, or whether those compounds were definitively accumulated in vacuoles and their movement back to the cytoplasm was impossible.

## MATERIALS AND METHODS

***Isolation of protoplasts and vacuoles.*** Protoplasts were isolated from leaves of *C. officinalis* by macerozyme and cellulase lysis as described earlier [9]. Vacuoles were liberated from protoplasts with DEAE-Dextran in isotonic conditions and were purified by centrifugation in discontinuous mannitol-sucrose-Ficoll gradient as described previously [1, 10].

***Radioactive precursors.*** 3-*O*-Monoglucoside and 3-*O*-monoglucuronide of [3-$^3$H]oleanolic acid were chemically synthesized as described earlier [11]. The obtained labelled compounds had a specific activity of 3.8 mCi/mmol.

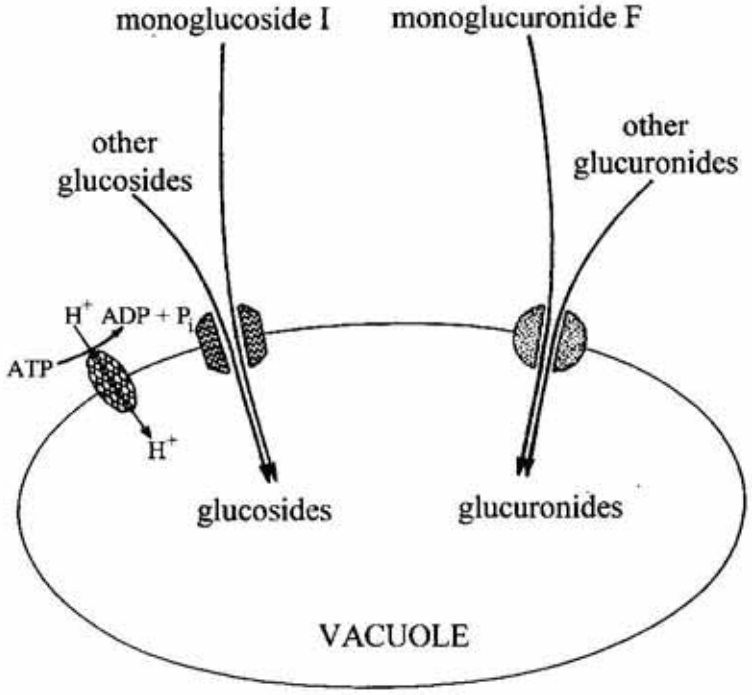


**Figure 2. Transport mechanisms of oleanolic acid glycosides to vacuoles in *Calendula officinalis* leaf protoplasts.**

***Administration of radioactive precursors.*** The incubation of isolated vacuoles with radioactive compounds ($9 \times 10^4$ d.p.m./$10^5$ vacuoles in 1 ml of incubation medium [12]) was carried out at an illumination of 3000 lux and temperature of 25°C for 20 min or, as a control, for 60 min with measurements every 10 min. The monoglycosides nonabsorbed into the vacuoles were washed off by centrifugation in the mannitol-sucrose-Ficoll gradient [1, 5].

***Efflux experiments.*** Vacuoles, purified after 20 min preincubation with radioactive

monoglycosides, were transferred either to the monoglycoside-free standard incubation medium or to the medium supplemented with a twofold excess of unlabelled monoglucoside or monoglucuronide (i.e. twofold higher concentrations with respect to that of radioactive I and F, respectively), for further 40 min incubation. Afterwards, the vacuoles were separated from the medium by being passed through a nylon filter. The number and viability of vacuoles was monitored at each experimental step by the neutral red staining method [1].

***Radioactivity measurements.*** The fractions of intact vacuoles and respective media were extracted with ethyl ether and n-butanol [1]. The radioactivity of monoglycosides was estimated in a Beckman scintillation counter.

## RESULTS AND DISCUSSION

The results of the efflux experiments, performed to check the possibility of reversible tonoplast transport, are summarized in Table 1. Vacuoles which had been preloaded with labelled monoglucoside I released about 5% of radioactivity during 40 min after the transfer into a fresh isotonic monoglycoside-free medium. Moreover, the accumulated monoglucoside I was lost in 55% after the transfer of preincubated vacuoles into a medium containing an excess of unlabelled compound. In contrast, vacuoles which had been preloaded with labelled monoglucuronide F, totally retained this compound when replaced in a fresh monoglycoside-free medium and did not release substantial amounts of radioactivity even in the medium containing an excess of unlabelled monoglucuronide F. The obtained results point to the possibility of reversible tonoplast transport of monoglucoside I, whereas monoglucuronide F seems to be definitively stored in the vacuolar space.

Further studies on the dynamics of the efflux (Fig. 3) fully supported those findings. Again, the addition of an excess of unlabelled monoglucoside I (Fig. 3I, the arrow) caused a rapid efflux of more than a half of preaccumulated radioactive compound, pointing to the possibility of reversible exchange between vacuolar and cytosolic pools of glucosides of oleanolic acid. In turn, it is well documented that some primary metabolites, mainly amino acids and sugars, which are only temporarily sequestered in the vacuole, are transported across the tonoplast by ATP-dependent systems [6]. So far, reversible tonoplast transport of compounds requiring energy to enter the vacuole, like oleanolic acid glucosides, has not been satisfactorily explained. Maybe other mechanism or even different carriers (or channels) are involved in the way back to the cytoplasm.

On the contrary, in the paralell experiment no monoglucuronide F efflux could be measured (Fig. 3F), so there is no evidence for reversible tonoplast transport of oleanolic acid glucuronides. Instead, such results indicate that the definitive vacuolar accumulation of monoglucuronide F can be a consequence of an ion-trap mechanism: a phenomenon regarded as analogous to immobilization of basic stains (e.g. neutral red) in-

**Table 1. The efflux of labelled oleanolic acid monoglycosides from preloaded vacuoles[a]**

| Compound | Radioactivity (d.p.m./$10^5$ vacuoles) | | | |
|---|---|---|---|---|
| | [$^3$H]Oleanolic acid monoglucoside | | [$^3$H]Oleanolic acid monoglucuronide | |
| | Inside the vacuoles | Outside the vacuoles | Inside the vacuoles | Outside the vacuoles |
| None | 17634 | 928 | 10086 | 60 |
| Oleanolic acid monoglucoside | 8117 | 9920 | n.d. | n.d. |
| Oleanolic acid monoglucuronide | n.d. | n.d. | 9950 | 85 |

[a]For details see Methods

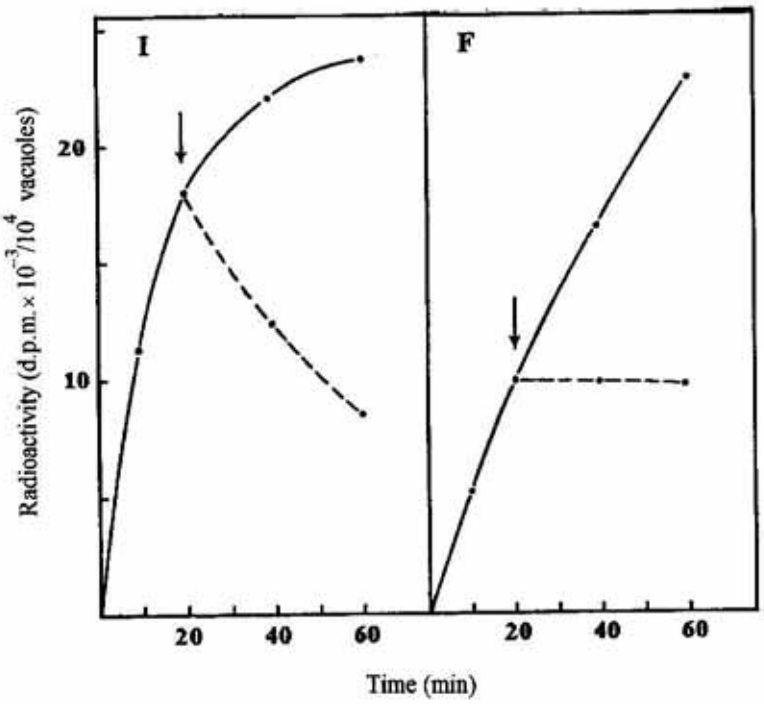


**Figure 3. The uptake (—) by isolated vacuoles and efflux (- - -) of oleanolic acid monoglucoside I and monoglucuronide F.**

Arrows indicate the time of transfer of aliquots of vacuoles to a medium containing an excess of unlabelled appropriate monoglycoside.

side vacuoles. The ion-trap mechanism is a good explanation for the vacuolar deposition of compounds which pass the tonoplast membrane by simple or carrier-mediated diffusion and have to be accumulated against a concentration gradient. In the acidic medium of the vacuole they can be either protonated and therefore trapped as cations, or immobilized by salt and complex formation with other vacuolar components, mainly phenolics [7, 13].

The above conclusions are in good accordance with our earlier hypothesis concerning the distinct difference in metabolic behaviour and physiological function of the two series of oleanolic acid glycosides. Glucosides, with their several times faster rate of biosynthesis, reversible transport to the vacuole and significant allelopatic activity [14, 15], are considered to form an active pool and a transport form of oleanolic acid in the *Calendula officinalis* plant. In contrast, glucuronides are probably typical secondary metabolites accumulating definitively in the vacuole and cell wall [10, 16].

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