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The first genistin absorption screening into vacuoles of *Trifolium pratense* L.

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ABSTRACT

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The determination of a transport mechanism for genistin (genistein-7-*O*-glucoside) across the tonoplast was performed on vacuoles from a cell culture of *Trifolium pratense* L. Genistin levels were examined in vacuoles as well as in an assay medium by HPLC (high-performance liquid chromatography) after treatment with various substances. MgATP increased the uptake of added genistin by 25%, but the nucleotide-free samples also contained this glycoside. Applying bafilomycin A1, an H⁺-ATPase inhibitor, indistinctly inhibited genistin absorption. However, vacuolar absorption of genistin was significantly reduced by N,N'-dicyclohexylcarbodiimide. This inhibitor can suppress both H⁺-ATPase and H⁺-PPase; the effect of pyrophosphate alone was not investigated. An increase in genistein levels, as result of genistin hydrolysis, could also affect the transport mechanism. The results investigated with inhibitors suggest the possible involvement of proton pumps; however additional research is required to confirm the participation of multidrug and toxin extrusion (MATE) proteins in genistin transport.

Keywords: red clover; in vitro culture; isoflavones; membrane transport; phytoestrogen

Red clover is well-known fodder and melliferous plant that is also used in modern phytotherapy (Sabudak and Guler 2009). This plant belongs to Fabaceae family, which is characterized by the presence of isoflavones, a flavonoid subgroup. Metabolites such as genistein (Gen), daidzein and their glycosides can be further metabolized (Klejdus et al. 2001). Thanks to their structure, these secondary metabolites are able to bind to estrogen receptor and partially affect relevant cells and processes; isoflavones are considered phytoestrogens (Albulescu and Popovici 2007). However, excessive consumption of clover and other fabaceous plants can cause urogenital disorders in cattle (Adams 1995). Isoflavones and their

metabolites may also enter cow's milk and affect its composition (Steinshamn 2010).

Flavonoids are mainly synthesized on the cytosolic membrane of the endoplasmic reticulum (Zhao and Dixon 2010), and they are stored in the vacuoles (Hernández et al. 2009) or exported out of the cells by various transport mechanism. ATP-binding cassette (ABC) transporters include a large and ubiquitous group of proteins that are composed primarily of two domains. Various substances are transported through transmembrane domains and the process is ensured by ATP hydrolysis on nucleotide-binding domains. Subgroup ABCB, ABCC and ABCG were determined in plants as secondary metabolite transporters (Rea 2007).

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Multidrug and toxin extrusion (MATE) proteins are mostly formed by 12 transmembrane α -helices. An electrochemical gradient serves as the source of energy for the transport (Omote et al. 2006). Proton pumps (H^+ -ATPase, H^+ -PPase) actively transport protons through membranes to sustain the gradient (Gaxiola et al. 2007). For example, MtMATE1 was identified as a flavonoids transporter in *Medicago truncatula* (Zhao and Dixon 2009).

The aim of this study was to determine transport mechanisms of genistin (Gin) absorption into red clover vacuoles. A vital part of isoflavone metabolism is process of its transport, which was observed in red clover cell cultures that produce secondary metabolites with various biological properties. By applying different compounds that impact transport processes, the uptake mechanism of added Gin could be hypothesized.

MATERIAL AND METHODS

Plant material cultivation. The Domoradice Plant Breeding Station provided *Trifolium pratense* cv. DO-8 seeds. Approximately 2 g of the callus, derived from seedlings, were used as an inoculum for a new culture. Cell mass was cultivated on a paper bridge immersed in Gamborg B5 medium (Gamborg et al. 1968) with 2,4-D (9.0 μ mol/L) and 6-BAP (8.8 μ mol/L; both SERVA, Heidelberg, Germany) growth regulators under stable conditions (24°C; 16 h light/8 h dark regime). The cell cultures used to isolate the vacuoles were derived by disrupting 5 g of the callus within the same liquid medium without the bridge. Cultivation in a Multi-Flask Shaker (115 rpm; VKS 75, Hechingen, Germany) ensured sufficient homogenization of the suspension. Cells were subcultured every 14 days by transferring 10 mL of thick suspension into 20 mL of fresh medium.

Isolation of protoplasts and vacuoles. A 10-day-old suspension culture was used for the isolation of protoplasts. The utilised method of vacuole isolation was adjusted according to Pomahacová et al. (2009). Cells were filtered and washed several times using 500 mmol/L of mannitol solution. 50 g of plant material was mixed with 100 mL solution of Cellulase Onozuka R-10 and Macerozyme R-10. A neutral red pigment was added to the mixture in order to visualize the protoplasts and vacuoles. The suspension was incubated along with

enzymes in the shaker (115 rpm) for 3–4 h until most protoplasts were released.

The released protoplasts (Figure 1a) were separated using a Mira cloth layer. The protoplast suspension was separated from the enzyme solution in a HERMLE Centrifuge Z326K (10 min, 800 g) and the resulting pellets were dispersed in a 20 mL solution containing 500 mmol/L of mannitol and 10 mmol/L of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The protoplast suspension was poured into test tubes and lysed with distilled water; repeated shaking bolstered release of the vacuoles.

The suspension was centrifuged once more (15 min, 4°C, 2650 g) and the pellets were dissolved in a standard solution (500 mmol/L mannitol, HEPES 10 mmol/L, 0.0375% PEG, 12.5 mmol/L EDTA (ethylenediaminetetraacetic acid)) containing 5% Ficoll. Vacuoles were thickened and cleaned using a step gradient with Ficoll solutions in equal parts (15%, 10% and the mixture containing 5% Ficoll and vacuoles). Upon separation (10 min, 4°C, 800 g) the vacuoles were removed from between the layers of Ficoll solutions (15% and 10%). The suspension was divided into 12 test tubes and centrifuged (15 min, 4°C, 2650 g).

Genistin transport assay. The pellets of vacuoles were diluted in test tubes with 8.0 mL of standard solutions that contained the tested substances (3.0 mmol/L MgATP; 0.03 mmol/L bafilomycin A1 – Baf; 0.5 mmol/L N,N'-dicyclohexylcarbodiimide – DCCD) with or without Gin (0.1 mmol/L); the other solutions contained only Gin or water as a blank sample. Immediately after preparation, 2.0 mL of the suspension was removed from each mixture of solutions and centrifuged (10 min, 4°C, 2650 g). The supernatant was mixed with 1.0 mL of 80% methanol and transferred to a vial. The pellet of vacuoles was dispersed in 2.0 mL of 80% methanol using the Labdancer for a duration of 15 s. The extract was filtered through a microfilter (0.2 μ m) into vials using an injection syringe. Sampling and processing were carried out in the same manner after 2, 4 and 24 h.

HPLC analysis. The Gin as well as the Gen content (Figure 1b) in the vacuoles and medium was established using HPLC analysis with a Jasco chromatography set (JASCO International, Tokyo, Japan) and the stationary phase LiChrospher 100 RP-18 column (5 μ m; Merck, Darmstadt, Germany) described in Kubeš et al. (2014). Individual isoflavone standards (genistin, genistein) were used to establish the results (Sigma-Aldrich, Schnellendorf, Germany).

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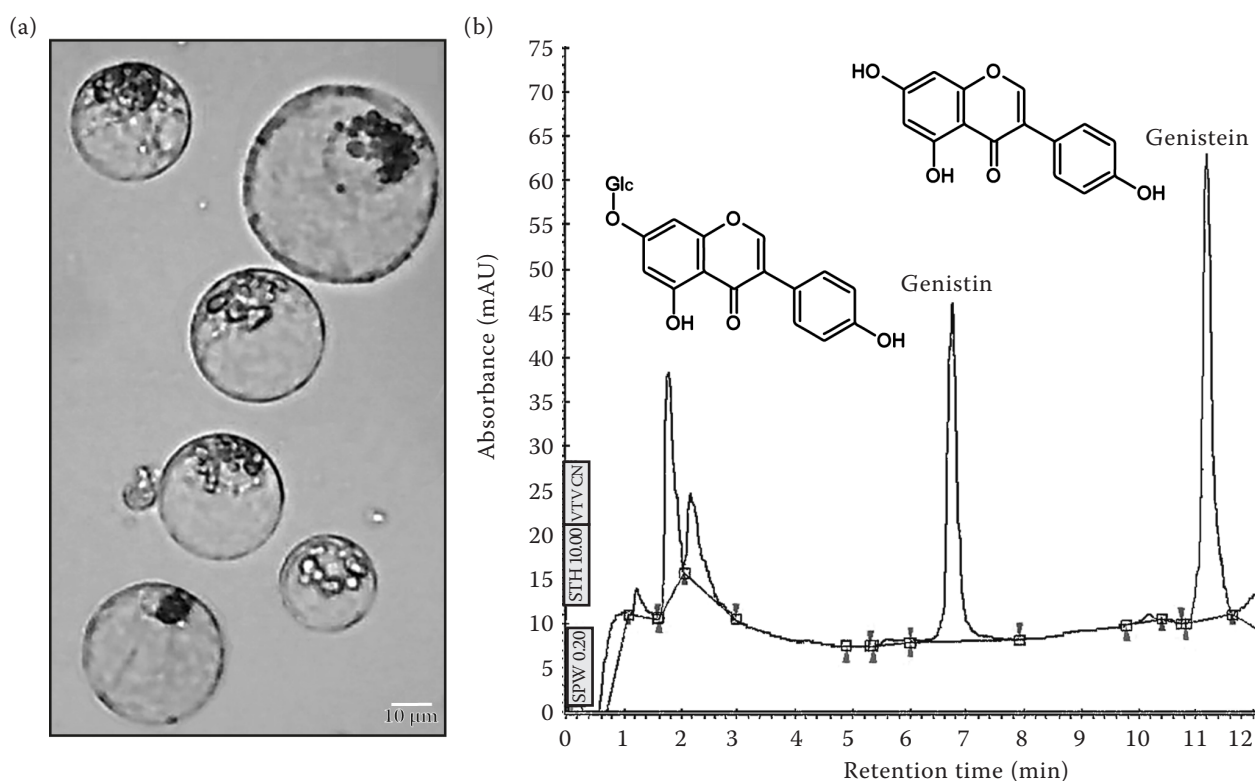


Figure 1. (a) Free protoplasts presented in the medium after 3 h in the enzyme solution. (b) Chromatogram of isoflavones in vacuoles after treatment with bafilomycin A1 (0 h)

The mobile phase contained methanol with a 0.15% phosphoric acid buffer. The flow rate in the column was 1.1 mL per min, and the methanol concentration gradually increased from 30% to 80% in the first 9 min. The column was eluted for 15 min using 80% methanol (an isocratic elution). Individual substances were detected using a MD-2015 Plus (JASCO International, Tokyo, Japan) diode array detector with a wavelength range of 190 nm to 450 nm. The isoflavone content was calculated from a peak wavelength value of 260 nm.

Statistical analysis. A mixed-model procedure, with a repeated statement for each parameter, was used to analyze the data set. Data from each measurement were tested separately. Tukey's test ($*P < 0.05$) was used to determine significant differences. All statistical analysis presented in this study were performed using Statistica 12 software (StatSoft Inc., Tulsa, USA).

RESULTS AND DISCUSSION

The transport mechanism description is an important part of specific transport protein iden-

tification. The combination of the transporter substrate and different effectors is used to clarify its transport processes. As discussed below, the content of added Gin in the vacuoles of red clover as well as in the assay medium was altered by these compounds. Overall, the application of effectors alone did not cause a significant difference in Gin levels in the vacuoles (data not demonstrated).

In line with previous studies, MgATP was the first substances to be tested. MgATP significantly increased the uptake of added Gin into the vacuoles (Figure 2A₁). Similar observation was reported by Sugiyama et al. (2007), who also described the significantly lower rate of isoflavone absorption when using other nucleotides (or their absence). However, the vacuolar samples only with Gin (time 0) also contained more glycoside compared to a blank sample (Figure 2A₁). As opposed to the samples with MgATP, Gin concentration was reduced by 25%. Nevertheless, the transport without MgATP was also described in the case of a glucoside of salicylic acid (SAG; 17%) in soybean (Dean and Mills 2004), as well as flavonoid glycosides saponarin (21%) and vitexin (70%) in barley (Frangne et al. 2002).

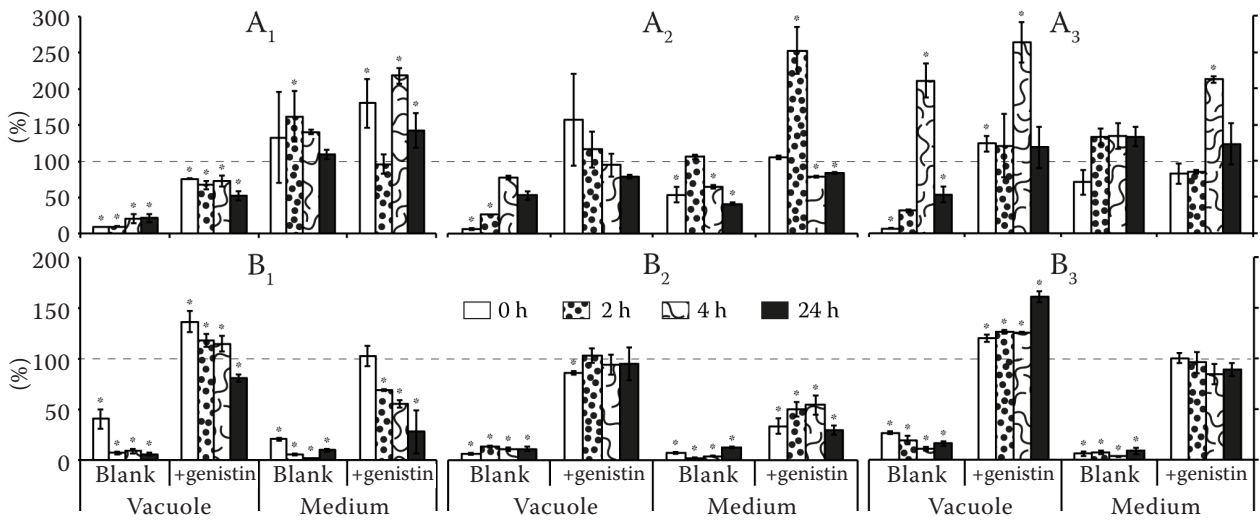


Figure 2. (A) Genistin and (B) genistein content in vacuoles and the medium after treatment with (1) MgATP; (2) bafilomycin A1, and (3) DCCD (N,N'-dicyclohexylcarbodiimide) during 24 h. All bar values were recalculated to a relative 100% isoflavone content in samples with the tested compound and genistin. ($x \pm$ standard deviation, $n = 3$; * $P < 0.05$, Tukey's test)

A positive effect of MgATP related to lower Gin levels was identified in the medium (time 0, 4, 24)

unlike the samples without MgATP (Figure 2A₁). In other time intervals during sampling, the con-

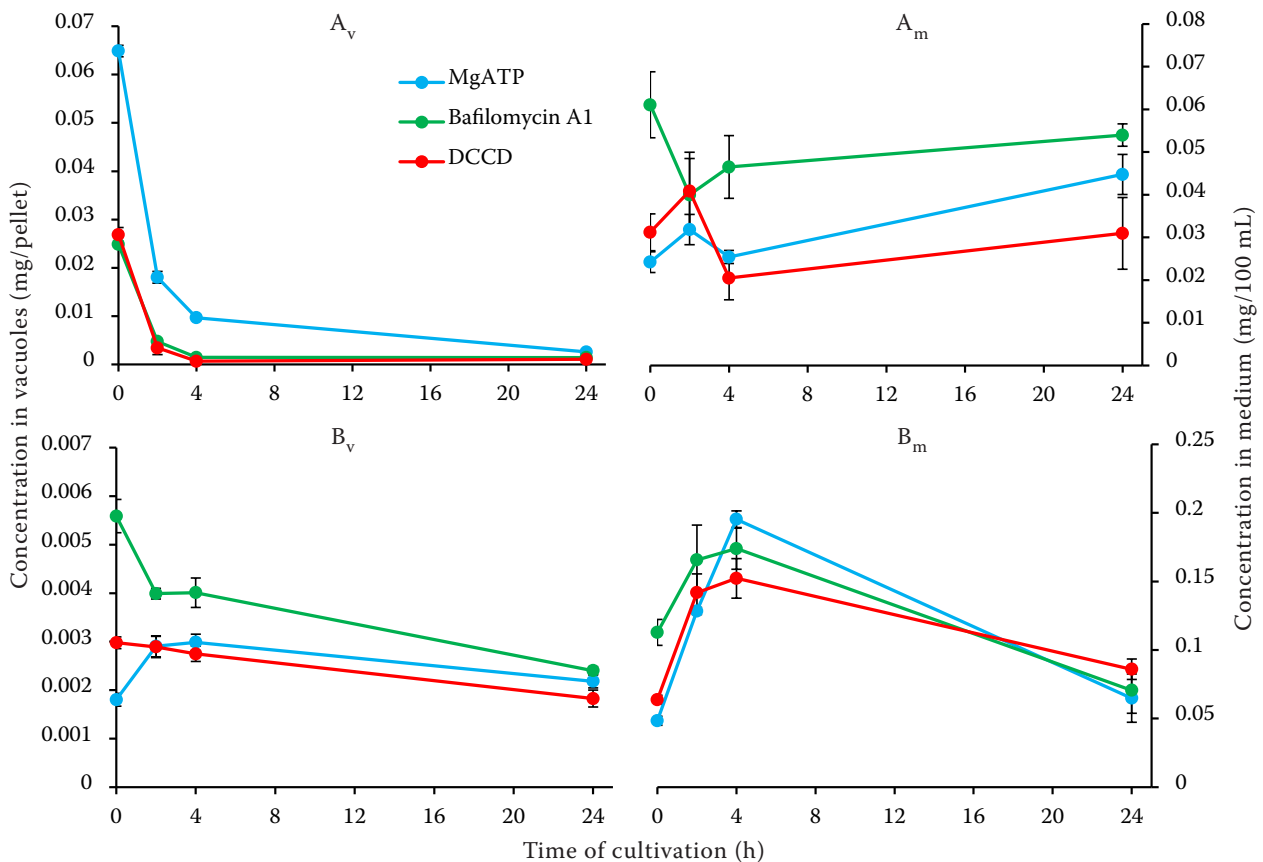


Figure 3. (A) Genistin and (B) genistein content in pellet of vacuoles (v) and the medium samples (m) with the tested compound + genistin ($x \pm$ standard deviation, $n = 3$)

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centration of glycoside in vacuoles gradually decreased and its content was variable in the case of the medium (Figure 3A_m). Studies using metabolites marked by an isotope can measure the course of absorption at shorter intervals; therefore, the independent process of Gin absorption could not be further identified here.

With regard to MgATP independent uptake of Gin, inhibitors of proton pumps were chosen as further transport process suppressors. The activity of P-ATPase or V-ATPase was inhibited by Baf according to its concentration; for example, the latter pump is a H⁺-ATPase of corn tonoplast (Bowman et al. 1988). The application of Baf caused that the vacuoles without inhibitor contained more of the added Gin (time 0), although this difference was insignificant. The content of Gin in samples without Baf, measured after 4 h or 24 h, was significantly lower in the medium. In spite of previous sampling, the inhibitor-free samples contained more Gin (Figure 2A₂).

A similar inhibition of flavonoids transport through tonoplast was associated with saponarin in barley (Frangne et al. 2002) as well as epicatechin 3'-O-glucoside in MATE1 in the case of *M. truncatula* (Zhao and Dixon 2009). However, MgATP was applied along with Baf in these studies and the suppressing effect of this inhibitor was more noticeable. By contrast, Baf affected ABC protein of bacteria *Salmonella typhimurium*, although the inhibitor concentration was a thousand times higher (Hunke et al. 1995).

In the case of DCCD, the inhibition of vacuolar H⁺-ATPase was also determined in *Acer pseudoplatanus* (Magnin et al. 1995). In regard to vacuoles of red clover, the content of added Gin was significantly higher in samples without this inhibitor immediately after preparation and after 4 h (Figure 2A₃). The content of Gin in a medium without the inhibitor was lower in time 0 h, but insignificant. A more visible inhibition effect of DCCD, compared to Baf, could be explained by the possible activity of DCCD against V-PPases, aside from H⁺-ATPases. Maeshima and Yoshida (1989) described that transport utilizing this pump in the tonoplast of mung bean (Fabaceae) depends on the hydrolysis of inorganic pyrophosphate. The presence of H⁺-PPases would explain the sustained absorption of Gin, because inhibitors such as Baf would only have a partial effect on the proton gradient disruption. Otherwise, even DCCD cannot fully inhibit transport, thanks to the possible

involvement of H⁺-ATPases that were stimulated by MgATP (Figure 2A₁).

Both inhibitors also had a similar effect on the content of added Gin in the vacuoles (Figure 3A_v). Szakiel and Janiszowska (2002) also indicated that possible combined activity by both types of proton pumps could not be excluded. The vacuolar transport of monoglucoside oleanolic acid, aside from Baf and other inhibitors with similar activity, was inhibited by DCCD in the presence of pyrophosphate in *Calendula* sp.

Genistin is a native glycoside of red clover and its presence was apparent in the blank samples (Figure 2a). It would also suggest the involvement of proton pumps and MATE transporter. The origin of substances and their structures affect absorption in favour of native glycosides. Unlike barley, saponarin is not present in *Arabidopsis thaliana*. Its vacuolar transport was not inhibited by Baf, but by orthovanadate, the inhibitor of ABC transporters (Frangne et al. 2002). Nonetheless, a transport using ABC transporters is possible even in tonoplast, as discussed in the case of soybean by Dean and Mills (2004).

Figure 3A_v indicates a gradual decrease of added Gin in vacuoles, independent on the studied effector. At the same time, several times higher concentration of its aglycone Gen was observed in the vacuole samples (Figure 2b) as well as in medium samples collected within the period of 2 h and 4 h (Figure 3B_m). However, the higher content of Gen in the medium was not accompanied by an analogical increase of concentration in the vacuoles in later observed samples. It could be supposed that the hydrolysis of Gin could take place separately in the vacuoles and the medium or uptake of aglycone was also reduced. Mackenbrock et al. (1992) described the presence of β-glucosidases in the cytoplasm and in cellular membranes, including the tonoplast of chickpea from the Fabaceae family. The presence of Gen in samples could be explained by the activity of these enzymes. Used solutions had pH > 5 excluding acid hydrolysis and they were added to the vacuoles shortly after preparation. Moreover, no hydrolysis of flavone glycoside baicalin was found with identical assay solutions within unpublished experiments on *Scutellaria baicalensis* vacuoles.

Nonetheless, the presence of Gen may explain an inconsistency in some results; aglycones and glycosides or metabolites with a similar structure

can negatively affect absorption by competition for a transport protein. Sugiyama et al. (2007) concluded that Gen absorption through the plasma membrane vesicles in soybean was most affected by daidzein and formononetin, less by Gin and minimally by flavanone naringenin and its glycoside. The high content of Gen in the medium as well as in vacuoles (Figures 2 and 3) could be explained as a result of negative effect on this glycoside absorption with increasing of Gin availability for the hydrolysis. On the other hand, the aglycone epicatechin failed to inhibit the transport of epicatechin 3'-O-glucoside in the case of *M. truncatula* (Zhao and Dixon 2009).

In regard to difficult determination of exact concentrations of the forming Gen, it is impossible to clarify in detail the possible process of its absorption. The amount of Gen was higher in the vacuoles and lower in the medium in samples without MgATP and with Gin (Figure 2B₁), when more glycoside was available for hydrolysis in the medium and the absorption of Gen was independent on MgATP.

Unlike Gen (Figure 2B₁), no transport through tonoplast occurred in the case of the pterocarpane medicarpin, either before or after the addition of MgATP. Glutathione was necessary for absorption and the transport was subsequently inhibited by vanadate. Proteins of the ABCC subgroup are responsible for transport of this way bonded substances then (Li et al. 1997, Rea 2007); such transport mechanism could be excluded. According to Dean and Mills (2004), salicylic acid was absorbed in a lesser amount compared to SAG in the presence of MgATP in soybean, but the transport of aglycone prevailed in nucleotide-free samples. The transport of aglycone in red beet was minimal in both assays.

The application of MgATP and two inhibitors within this study affected Gin presence in the vacuoles to varying degrees that could be linked to the mechanism dependent on proton gradient. In the past, a number of studies indicated that native flavonoids are transported by the MATE proteins. However, the results of the isoflavone glycoside daidzin absorption in the case of *M. truncatula* did not exclude the possible additional involvement of an ABC transporter (Zhao and Dixon 2009). Szakiel and Janiszowska (2001) also discussed a passive transport, because proton pumps or ABC transporters inhibitors had no effect on the monoglucuronide oleanolic acid uptake.

Recent studies focusing exclusively on the vacuolar absorption of isoflavones similar to this study have not yet been published; while around 40 possible MATE proteins were identified (Zhao and Dixon 2009). Aside from the potential involvement of the transporters discussed here, the possible transport of flavonoids could include vesicles (Zhao and Dixon 2010). The possibility to verify this mechanism, as well as the effects of other substances (vanadate, NH₄Cl, various isoflavones), suggests future steps in research of Gin transport into the red clover vacuoles.

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