Different Energization Mechanisms Drive the Vacuolar Uptake of a Flavonoid Glucoside and a Herbicide Glucoside*

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Glycosylation of endogenous secondary plant products and abiotic substances such as herbicides increases their water solubility and enables vacuolar deposition of these potentially toxic substances. We characterized and compared the transport mechanisms of two glucosides, isovitexin, a native barley flavonoid C-glucoside and hydroxyprimisulfuron-glucoside, a herbicide glucoside, into barley vacuoles. Uptake of isovitexin is saturable (Kₘ = 82 μM) and stimulated by MgATP 1.3-1.5-fold. ATP-dependent uptake was inhibited by bafilomycin A₁, a specific inhibitor of vacuolar H⁺-ATPase, but not by vanadate. Transport of isovitexin is strongly inhibited after dissipation of the ΔpH or the ΔΨ across the vacuolar membrane. Uptake experiments with the heterologue flavonoid orientin and competition experiments with other phenolic compounds suggest that transport of flavonoid glucosides into barley vacuoles is specific for apigenin derivatives. In contrast, transport of hydroxyprimisulfuron-glucoside is strongly stimulated by MgATP (2.5-3 fold), not sensitive toward bafilomycin, and much less sensitive to dissipation of the ΔpH, but strongly inhibited by vanadate. Uptake of hydroxyprimisulfuron-glucoside is also stimulated by MgGTP or MgUTP by about 2-fold. Transport of both substrates is not stimulated by ATP or Mg²⁺ alone, ADP, or the nonhydrolyzable ATP analogue 5'-adenyl-β,γ-imidodiphosphate. Our results suggest that different uptake mechanisms exist in the vacuolar membrane, a pH-dependent uptake mechanism for specific endogenous flavonoid-glucosides, and a directly energized mechanism for abiotic glucosides, which appears to be the main transport system for these substrates. The herbicide glucoside may therefore be transported by an additional member of the ABC transporters.

In plants, secondary products such as species-specific phenolics as well as foreign compounds are often glycosylated in order to increase their water solubility. Storage of these compounds within the large central vacuole, a compartment with a very low metabolic activity, is generally assumed to protect the plant cell against potentially toxic effects of these substances, especially of their non-glycosidic form. Corresponding processes exist for the excretion of toxic conjugates in animals, e.g. at the canalicular membrane of liver hepatocytes (1, 2). Transport mechanisms of some alkaloids and phenylpropane derivatives have been suggested to be species- and substrate-specific (3–5). However, vacuolar storage of glucosylated abiotic substances such as 2,4-dichlorophenoxyacetic acid or transport of hydroxyprimisulfuron-glucoside as well as uptake of phenolic compounds not present in the considered species into vacuoles have been demonstrated (6–8). These observations indicate that the vacuole has the potential to detoxify and store not only endogenous but also foreign, biotic, and abiotic glycosylated substances.

Besides glycosylation, further metabolic modifications of the basic C₁₅ structure of flavonoids are well known and include oxidation, hydroxylation, methylation, and acylation reactions (9), leading to the giant chemical diversity within this class of natural phenolic compounds. The respective ligands may play a role in substrate recognition of the corresponding, species-specific, vacuolar transporter. In the case of Petroselinum hortense and Daucus carota cell cultures, it has been shown that acylation of apigenin 7-O glucoside and an anthocyanin, respectively, was a prerequisite for uptake into vacuoles (5, 10) and that protonophores inhibited uptake of the anthocyanin into Daucus vacuoles (5). Different strategies may be involved in vacuolar accumulation of coumaric acid glucosides: (i) vacuolar uptake of α-coumaric acid glucoside occurring in Mellilotus alba was shown to be independent of ATP, but involved an isomerization of the trans- to the vacuolar deposited cis-isomer (11). (ii) Esculin, a coumaric acid glucoside occurring in, e.g. potato but not in barley, was transported into barley vacuoles in an ATP-dependent, protonophore-sensitive manner, suggesting the existence of a proton antiport mechanism (7).

Recently, Marrs et al. (12) demonstrated that the gene characterized by the bronze-2 mutation, which is the last genetically defined step in anthocyanin biosynthesis in maize, encodes a glutathione S-transferase. They provided evidence that vacuolar deposition of the maize anthocyanin occurs via an intermediary, but yet unidentified, glutathione conjugate. From previous results showing the presence of an energized glutathione-conjugate ATPase (13, 14), it was suggested that anthocyanins may be accumulated within the vacuole via such a pump.

Little information is available on the transport of glucosylated xenobiotics. We have shown that the herbicide glucoside hydroxyprimisulfuron-glucoside is transported into vacuoles of barley and that this transport is stimulated by safeners, substances conferring tolerance toward herbicides (8). However,
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Vaccular accumulation of different compounds can be driven by the H⁺-ATPase or H⁺-pyrophosphatase via an H⁺ antiport mechanism (e.g. Na⁺/H⁺, Ca²⁺/H⁺), as a response to ΔΨ (e.g. malate) (15) or directly by ATP as demonstrated for glutathione-conjugates (13, 14) and bile acids (16). Calculations based on ΔG° values of ATP hydrolysis assuming cytosolic ATP, ADP, and P, concentrations as published demonstrate that a much higher vacuolar accumulation can be expected by a directly energized glutathione-conjugate pump compared with secondary energized transport mechanisms (17).

There are many open questions concerning the energization of glycosylated phenolic substances, and no information is available on the transport of glycosylated xenobiotics. Therefore it was of interest to investigate (i) whether species-specific and/or general vacuolar transport mechanisms exist for endogenous and foreign glucosides in the same plant and (ii) which type of energization is used by the respective transport system.

EXPERIMENTAL PROCEDURES

Barley (Hordeum vulgare L. cv. Bakara) was grown as described (8). Mesophyll cell protoplasts and vacuoles were isolated from 10-day-old primary leaves following a procedure of Rentch and Martinova (18). [14C]Isovitexin and [14C]orientin were prepared by feeding 40 5-day-old, young growth stage coryledons (Pogonomyrum ecelentum Moench cv. Prego) with 10 ml of aqueous solution containing 294 kBq × μmol⁻¹ l⁻¹[14C]phenylalanine (DuPont NEN) as described (19). After 120 h of incubation the phenylpropanoid, compounds were extracted with 80% (v/v) MeOH (20), and the solution was evaporated and redisolved in MeOH. Separation of buckwheat flavonoids was performed on a preparative RP-18 column (30 cm × 16 mm) connected to a low pressure pump (Duramat CFG Prominent, Chemie und Filter GmbH, Heidelberg, Germany). The RP-18 column was preconditioned with 100 ml of 10% (v/v) acetic acid and washed with 300 ml of H2O. Phenylpropanes were eluted by raising the methanol concentration stepwise (0, 5, 10, 12.5, 15, 17.5, 20% (v/v); 200 ml each). At 22.5% (v/v) methanol (800 ml) orientin and isovitexin were eluted and separated showing specific activities of 7.9 and 2.0 Bq × μmol⁻¹, respectively, and a purity of >98% as judged from HPLC analysis performed on a Shimadzu HPLC System (Shimadzu, Tokyo, Japan) under the following conditions: Nucleosil RP-18 column (125 × 4.6 mm; 5 μm grain size; CS Chromatographie, Langerwehe, Germany), constant flow rate of 1 ml min⁻¹, solvent 12% (v/v) acetonitrile in water containing 1% orthophosphoric acid, detection 220 nm. Purity was also checked at detection wavelengths 280 nm (data not shown). The identity of the buckwheat flavonoids and of the major barley flavonoid was further verified by co-chromatography and spectral analysis of the corresponding HPLC peaks with authentic substances kindly provided by Profs. H. Geiger and R. Mues (University of Saarbrücken, Germany) or purchased from Roth (Karlsruhe, Germany).

Hydroxyprimisulfuron-[14C]glucoside (0.37 MBq × μmol⁻¹) was synthesized enzymatically from 5-hydroxyphenylprimisulfuron and UDP-[U-14C]glucose using partially purified glucosyltransferase from Fagopyrum esculentum Moench cv. Prego with 10 ml of aqueous solution containing 294 kBq × μmol⁻¹ l⁻¹[14C]phenylalanine (DuPont NEN) as described (19). After 120 h of incubation the phenylpropanoid, compounds were extracted with 80% (v/v) MeOH (20), and the solution was evaporated and redisolved in MeOH. Separation of buckwheat flavonoids was performed on a preparative RP-18 column (30 cm × 16 mm) connected to a low pressure pump (Duramat CFG Prominent, Chemie und Filter GmbH, Heidelberg, Germany). The RP-18 column was preconditioned with 100 ml of 10% (v/v) acetic acid and washed with 300 ml of H2O. Phenylpropanes were eluted by raising the methanol concentration stepwise (0, 5, 10, 12.5, 15, 17.5, 20% (v/v); 200 ml each). At 22.5% (v/v) methanol (800 ml) orientin and isovitexin were eluted and separated showing specific activities of 7.9 and 2.0 Bq × μmol⁻¹, respectively, and a purity of >98% as judged from HPLC analysis performed on a Shimadzu HPLC System (Shimadzu, Tokyo, Japan) under the following conditions: Nucleosil RP-18 column (125 × 4.6 mm; 5 μm grain size; CS Chromatographie, Langerwehe, Germany), constant flow rate of 1 ml min⁻¹, solvent 12% (v/v) acetonitrile in water containing 1% orthophosphoric acid, detection 220 nm. Purity was also checked at detection wavelengths 280 nm (data not shown). The identity of the buckwheat flavonoids and of the major barley flavonoid was further verified by co-chromatography and spectral analysis of the corresponding HPLC peaks with authentic substances kindly provided by Profs. H. Geiger and R. Mues (University of Saarbrücken, Germany) or purchased from Roth (Karlsruhe, Germany).

Hydroxyprimisulfuron-[14C]glucoside (0.37 MBq × μmol⁻¹) was synthesized enzymatically from 5-hydroxyphenylprimisulfuron and UDP-[U-14C]glucose using partially purified glucosyltransferase from maize with a radiochemical purity of 97% as judged by thin-layer chromatography.

Uptake of [14C]isovitexin, [14C]orientin, and hydroxyprimisulfuron-[14C]glucoside into barley mesophyll vacuoles was measured as described (8, 13). For each condition and time point, polyethylene microcentrifugation tubes (400 μl) were prepared as follows: 70 μl of incubation medium containing 23% (v/v) Percoll, 0.4 μM solbitol, 30 mM KCl, 25 mM Hepes-KOH, pH 7.2, 0.12% bovine serum albumin, 0.55 kBq of [14C]isovitexin, 0.46 kBq of [14C]orientin, or 1.85 kBq of hydroxyprimisulfuron-[14C]glucoside, 3.7 kBq of 3H2O, and further solutes as indicated in the figures and tables were placed on the bottom of the tube. Uptake was started by addition of 30 μl of vacuole suspension. The samples were rapidly overlaid with 200 μl of silicone oil AR 200 (Fluka, Buchs, Switzerland) and 60 μl of water. The incubation was terminated by centrifugation at 10,000 × g for 15 min. Radioactivity was determined by liquid scintillation counting in 50 μl of the upper aqueous phase containing the hypotonically shocked vacuoles. 3H2O was used to calculate the vacuolar volume as it equilibrates rapidly between the medium and the vacuolar lumen.

Unless stated otherwise, uptake studies with unlabeled isovitexin were performed with 0.1 mM isovitexin in the above mentioned incubation medium with six tubes per condition and time point. After centrifugation the aqueous supernatants of three tubes were pooled, lyophilized, and redisolved in 100–200 μl of 80% (v/v) methanol before HPLC analysis. Separation of buckwheat flavonoids was performed on the Shimadzu HPLC system using a Nucleosil RP-18 column (125 mm × 4.6 mm; 5 μm grain size; CS Chromatographie, Langerwehe, Germany) and a gradient system described previously (21). Uptake was calculated based on the total peak area of the major flavonoid apigenin showing no change in its concentration during the time course of the experiments (see “Results”) or based on the 3H2O counts measured in separate assays. Unless stated otherwise, uptake rates of hydroxyprimisulfuron-[14C]glucoside were calculated by subtracting the radioactivity measured after 2 min of incubation from the 20-min values and uptake rates of unlabeled isovitexin by subtracting the vacuolar isovitexin/saponarin ratio after 2 min from the corresponding 15-min value. Ka and Vmax values were calculated using a computer program (Enzfitter, Biosoft, Cambridge, United Kingdom).

RESULTS

Plants contain a variety of glycosylated endogenous compounds and are able to glucosylate abiotic substances as a possible step in the detoxification of xenobiotics. For both groups of glucosides vacuolar localization is generally assumed. In an attempt to compare the transport specificities of an abiotic glucoside with two plant-borne glucosides across the vacuolar membrane, we used hydroxyprimisulfuron-[14C]glucoside (Fig. 1C); isovitexin (apigenin 6-C-glucoside, Fig. 1A) and orientin (luteolin 8-C-glucoside, Fig. 1B). Uptake of 380 μM [14C]isovitexin, a minor barley flavonoid, was linear with time for about 15 min and slightly stimulated by MgATP, while the luteolin derivative orientin at 220 μM, occurring, e.g. in buckwheat but not detectable in barley primary leaves, was obviously excluded from the vacuole (Fig. 2A). As the incorporation rate of [14C]label from phenylalanine into the buckwheat flavonoids was not sufficient for large scale experiments, isovi-

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1. The abbreviations used are: HPLC, high performance liquid chromatography; ABC, ATP-binding cassette; AMP-PNP, 5′-adenyl-β,γ-imidodiphosphate; HPS-glucose, hydroxyprimisulfuron-glucoside.
2. K. Kreuz, unpublished results.
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Isolated barley vacuoles were incubated in the presence of 100 μM hydroxyprimisulfuron-[14C]glucoside (HPS-glucose) or 100 μM isovitexin, and the compounds listed in the table. 100% MgATP-stimulated uptake corresponds to a rate of 6.87 ± 2.17 × 10⁻³ mol of isovitexin × s⁻¹ × liter⁻¹ and 4.32 ± 1.21 × 10⁻³ mol of hydroxyprimisulfuron-glucoside × s⁻¹ × liter⁻¹. Values are means of three to six independent experiments, when S.D. values are given or from two experiments (without S.D.). Each single experiment consisted of five replicates for hydroxyprimisulfuron-glucoside and two replicates for isovitexin uptake. For details see "Experimental Procedures." Uptake rates of hydroxyprimisulfuron-[14C]glucoside were calculated by subtracting the radioactivity measured after 2 min from the 20-min values and uptake rates of unlabeled isovitexin by subtracting the isovitexin/saponarin ratio after 2 min from the corresponding 15-min value. ND, not determined.

| Treatment | Transport of |
|-----------|--------------|
| HPS-glucose | Isovitexin |
| % of MgATP-stimulated uptake | |
| 1 mM Mg²⁺ | 32.3 ± 8.8 | 73.3 ± 6.9 |
| 4 mM Mg²⁺ | 23.0 | 67.8 ± 4.2 |
| 3 mM ATP (control) | 100 | 100 |
| 3 mM ATP | 35.2 ± 12.0 | 69.3 ± 4.8 |
| 3 mM UTP | 4 mM Mg²⁺ | 69.0 | 66.0 ± 2.1 |
| 3 mM GTP | 4 mM Mg²⁺ | 60.3 ± 7.9 | 47.1 ± 3.3 |
| 3 mM AMP-PNP | 4 mM Mg²⁺ | 32.0 | 71.4 ± 0.3 |
| 3 mM ADP | 4 mM Mg²⁺ | ND | 57.7 ± 0.4 |
| 0.2 mM PP | 4 mM Mg²⁺ | 35.0 | 58.8 ± 2.6 |
| 10 mM UDP-glucose | 1 mM Mg²⁺ | ND | 69.8 ± 7.3 |

Table I

Effect of different nucleotides and pyrophosphate on the uptake of isovitexin and hydroxyprimisulfuron-glucoside and of UDP-glucose on the uptake of isovitexin into barley vacuoles

Transport of isovitexin was studied using unlabeled substrate and detection of the flavonoid by HPLC. Vacuolar and whole leaf extracts of *Hordeum vulgare* L. cv. Bakara contain only trace amounts of isovitexin being the precursor of saponarin (isovitexin 7-O-glucoside) (22), which accumulates as the major compound (Fig. 2B). Unlabeled isovitexin is readily taken up by barley vacuoles with a time dependence and MgATP stimulation comparable with the results obtained using [14C]isovitexin. The fact that only the isovitexin peak increases with time (Fig. 2B), while the amounts of all other detected compounds remain constant, suggests that neither isovitexin is converted into saponarin via glucoylation nor saponarin is deglucosylated to isovitexin during the course of the experiment. This observation is further supported by the fact that the uptake rate did not change when barley vacuoles were incubated in the presence of 100 μM isovitexin together with 10 mM UDP-glucose (Table I). The saponarin content (0.42 ± 0.09 × 10⁻³ mol × liter⁻¹ vacuolar volume, mean of 10 determinations) can therefore be used as a marker for vacuolar space in isovitexin uptake experiments in addition to H₂O volume determined in separate assays. The transport rates calculated using the two parameters proved to be identical within experimental error.

Uptake of the abiotic hydroxyprimisulfuron-[14C]glucoside (Fig. 1C) by barley mesophyll vacuoles has been shown to be linear for at least 20 min in a previous publication (8).

Transport of isovitexin is a saturable process with an apparent Km value of 82 ± 38 μM and Vmax of 6.09 ± 1.66 × 10⁻³ mol of isovitexin × s⁻¹ × liter⁻¹ (n = 5) (data not shown). In contrast, the hydroxyprimisulfuron-[14C]glucoside exhibits a linear concentration dependence up to 250 μM (data not shown), indicating the presence of a low affinity transport system.

ATP-dependent stimulation of the herbicide glucoside and the flavonoid glucosides is remarkably different: while isovitexin uptake is stimulated 1.4-fold, a 2.5–3-fold increase of the uptake rate can be observed for the herbicide glucoside (Table I). However, the MgATP-stimulated transport rates of hydroxyprimisulfuron-glucoside and isovitexin (4.32 ± 1.21 ×
10^{-5} \text{ mol of hydroxyprimisulfuron-glucoside} \times s^{-1} \times \text{liter}^{-1} \text{ and } 6.87 \pm 2.17 \times 10^{-7} \text{ mol of isovitexin} \times s^{-1} \times \text{liter}^{-1} \text{ both at } 100 \mu M, \text{ respectively, indicate a } 10-20\text{-fold higher flux of the flavonoid compared to the xenobiotic glucoside. The ratio of the uptake rate of isovitexin in the presence or absence of ATP does not increase at lower concentrations (1.2-fold at 20 \mu M isovitexin), suggesting that the protons transferred by the vacuolar H^+\text{-ATPase} are not a limiting step in energization. Incubation of vacuoles for 15 min with 100 \mu M isovitexin results in vacuolar concentrations of 380 \pm 150 in the absence and 560 \pm 190 \mu M isovitexin in the presence of MgATP (n > 7), demonstrating uptake against the concentration gradient. Mg^{2+} \text{ or ATP alone as well as ADP are not able to stimulate uptake of both substrate, and the nonhydrolyzable ATP analogue AMP-PNP cannot substitute for ATP, suggesting that ATP hydrolysis is a prerequisite for transport energization (Table I). In the case of hydroxyprimisulfuron-glucoside, but not for isovitexin, UTP and GTP can partially substitute for ATP hydrolysis is a prerequisite for transport energization (Table II). MgATP-stimulated transport of the herbicide glucoside is not inhibited by baflomycin A1, a highly specific inhibitor of the vacuolar membrane H^+\text{-ATPase (23). The uptake of isovitexin decreases in the presence of this antibiotic to levels observed in the absence of ATP. Vanadate, an inhibitor acting as a phosphate analogue, strongly decreases the uptake of hydroxyprimisulfuron-glucoside, but not of isovitexin. The transport of both glucosides is not inhibited by azide, an effector of F-type ATPases. A different behavior of the two transport systems is also observed after the addition of NH_4Cl, which dissipates the ΔH across the membrane, resulting in a strongly inhibited isovitexin transport, whereas hydroxyprimisulfuron-glucoside is only slightly affected in the presence of MgATP. However, addition of NH_4Cl in the absence of MgATP reduces the uptake rate of the herbicide glucoside to about 44% of the control without ATP. Thus, a nonenergized component of herbicide glucoside uptake may be affected by protonophores in contrast to the major component observed in the presence of ATP. Valinomycin, a K^+ specific ionophore, has an inhibitory but yet weaker effect on flavonoid glucoside uptake compared with the effect of the ΔH-dissipating reagent. Surprisingly, saponarin efflux is not observed after destroying ΔH or ΔΨ (data not shown).}

Differences in the transport mechanisms of the two glucosides can also be observed using inhibitors of ATPases and reagents affecting ΔH or ΔΨ (Table II). MgATP-stimulated transport of the herbicide glucoside is not inhibited by baflomycin A1, a highly specific inhibitor of the vacuolar membrane H^+\text{-ATPase (23). The uptake of isovitexin decreases in the presence of this antibiotic to levels observed in the absence of ATP. Vanadate, an inhibitor acting as a phosphate analogue, strongly decreases the uptake of hydroxyprimisulfuron-glucoside, but not of isovitexin. The transport of both glucosides is not inhibited by azide, an effector of F-type ATPases. A different behavior of the two transport systems is also observed after the addition of NH_4Cl, which dissipates the ΔH across the membrane, resulting in a strongly inhibited isovitexin transport, whereas hydroxyprimisulfuron-glucoside is only slightly affected in the presence of MgATP. However, addition of NH_4Cl in the absence of MgATP reduces the uptake rate of the herbicide glucoside to about 44% of the control without ATP. Thus, a nonenergized component of herbicide glucoside uptake may be affected by protonophores in contrast to the major component observed in the presence of ATP. Valinomycin, a K^+ specific ionophore, has an inhibitory but yet weaker effect on

### Table II

**Vacuolar Uptake of Flavonoid and Herbicide Glucosides**

| Treatment | HPS-glucose | Isovitexin | % of MgATP-stimulated uptake |
|-----------|-------------|-----------|------------------------------|
| Control   | 100         | 100       | 100%                          |
| +Baflomycin A1 (0.1 μM) | 114.0 ± 16.0 | 79.3 ± 5.7 | 35%                           |
| +Vanadate (0.2 mM) | 42.6 ± 7.8 | 104.9 ± 16.2 | 20%                           |
| +NaNO_3 (1 mM) | 82.0 | 92.5 ± 0.5 | 100%                          |
| +NH_4Cl (5 mM) | 87.0 ± 10.0 | 98.1 ± 14.8 | 95%                           |
| +Valinomycin (5 μM) | ND | 64.9 ± 7.6 | 65%                           |

### Table III

**Influence of potential competitive inhibitors, flavonoid-glucosides, and other glucosylated substances, on the uptake of isovitexin and hydroxyprimisulfuron-glucoside**

| Treatment | Derivative of HPS-glucose | Isovitexin | % of control |
|-----------|---------------------------|-----------|-------------|
| Control   | Apigenin                  | ND        | 100         |
| +Saponarin| Apigenin                  | ND        | 41.9 ± 17.6 |
| +Apigenin 7-O-glucoside | Apigenin | 17.7 ± 1.5 | 0.0 ± 4.7   |
| +Vitexin  | Apigenin                  | 73.1 ± 4.6| 51.4 ± 5.6  |
| +Isovitexin| Apigenin                  | 44.6 ± 1.2| 98.1        |
| +Lutonarin| Luteolin                  | ND        | 125.3       |
| +Quercetin| Luteolin                  | ND        | 74.7 ± 6.0  |
| +Sinigrin | Glucosinolate             | 81.0      | ND          |
| +Arbutin  | Simple phenol             | 84.9 ± 6.9| 95.0 ± 9.5  |
| +Naringin | Coumarin                  | 60        | 86.6 ± 3.5  |
| +Methylumbelliferyl glucoside | Coumarin | 95.0 ± 9.5 | 81.7 ± 8.8  |
| +β-Methylumbelliferyl glucoside | Coumarin | 70.4 ± 3.9 | 39.3 ± 5.9  |

Isolated vacuoles were incubated in the presence of 40 μM HPS-glucose or isovitexin and further compounds as indicated. Due to the limited solubility of phenolic substances in aqueous solution, external concentrations of the glucosides were 41 μM for vitexin and 120 μM for all other substances. 100% correspond to an uptake rate of 2.43 ± 0.36 × 10^{-5} mol of HPS-glucose × s^{-1} × liter^{-1} and 1.82 ± 0.41 × 10^{-5} mol of HPS-glucose × s^{-1} × liter^{-1}. Uptake experiments with isovitexin were performed in the presence of 1 mM Mg^{2+}. In competition experiments with HPS-glucose, flavonoid competitors were added at 120 μM while all other glucosides were added at 1 mM, since preliminary experiments with 120 μM showed no inhibition. Uptake of the HPS-glucose was measured in the presence of 3 mM ATP and 4 mM Mg^{2+}. Values are means of 2 (no S.D. values) to 3 (S.D. values given) independent experiments. Each experiment consists of five replicates for hydroxyprimisulfuron-glucoside and two replicates for isovitexin uptake. For calculation of uptake rates see the legend to Table I. For saponarin competition of isovitexin uptake the values calculated from the 3H2O volume were used. ND, not determined.
take of hydroxyprimisulfuron-glucoside to about 80%, while vitexin has a much lower effect. In addition, isovitexin itself reduces uptake of the herbicide glucoside by about 55%. Other glucosides, which were in contrast to isovitexin experiments added at a final concentration of 1 mM, inhibit transport of the abiotic compound between 5 and 40% with esculin being the most inhibitory. In correspondence to the results obtained for isovitexin, the β-isomer of methylumbelliferyl glucoside has a more pronounced inhibitory effect on the uptake of hydroxyprimisulfuron-glucoside than the isomer bearing the glucose residue in the α-position. However, β-methylumbelliferyl glucoside decreases the transport of the herbicide glucoside only by about 30%, while isovitexin uptake is stronger affected by this compound (60% inhibition).

**DISCUSSION**

Discrepancies exist between the observation that investigations using plant-specific secondary products exhibited species-specific uptake into vacuoles of the corresponding substance and the description of heterologous or even abiotic substances entering the vacuole (3–5, 7, 8). The goal of our investigation was to elucidate whether two different glucosides taken up by barley vacuoles use identical or different transport systems and which energization mechanisms are involved.

Our results strongly suggest that two different mechanisms exist for the transport of the flavonoid isovitexin (apigenin 6-C-glucoside, Fig. 1A) and of a herbicide glucoside (hydroxyprimisulfuron-glucoside, Fig. 1C) for the following reasons: (i) uptake of hydroxyprimisulfuron-glucoside is quite slow under nonenergized conditions but is stimulated 2.5–3-fold in the presence of MgATP. In contrast, isovitexin uptake exhibits a much higher absolute uptake rate, while the addition of MgATP has only a slight but reproducible effect (Fig. 2B and Table I). (ii) There are remarkable differences in nucleotide specificities, since UTP and GTP can partially substitute for ATP only in the case of the herbicide glucoside (Table I). The observation that GTP rather inhibits flavonoid uptake is not understood yet. (iii) Transport of isovitexin and hydroxyprimisulfuron-glucoside is differentially affected by inhibitors specific for different types of ATPases.

Based on these observations we propose that two glucoside transporter exist differing in their energization mechanism. Isovitexin transport by barley vacuoles is driven by the ΔpH, while at least 60% of hydroxyprimisulfuron-glucoside uptake is mediated by a directly energized carrier.

The herbicide glucoside carrier may be a novel member of the transport ATPases belonging to the ABC family, which have been found during the last years in animals, fungi, and plants. ABC transporters are thought to be mainly involved in the cellular detoxification mechanisms (24). However, for many of these ABC transporters only the respective gene is known, while the corresponding substrate is unknown (25). For plants it has been shown that two different transport activities reside in the vacuolar membrane, which are directly energized by ATP: the transfer of glutathione S-conjugates (13, 14) and of bile acids (16). These carriers accumulate their substrate in the vacuole, even if the vacuolar H+-ATPase is inhibited by bafilomycin and the ΔpH abolished by NH₄Cl. In the case of hydroxyprimisulfuron-glucoside, the uptake rates were too low to proof accumulation against a concentration gradient. However, the general properties are very similar as compared with the other described directly energized transporters: requirement for MgATP; GTP and UTP can partially substitute for ATP; transport is not inhibited by bafilomycin and NH₄Cl but by vanadate. Therefore we suggest that the MgATP-dependent component of the hydroxyprimisulfuron-glucoside transport activity occurs by a directly energized ATPase.

So far, transport of species-specific flavonoids across the vacuolar membrane has only been studied for acylated flavonoid glucosides (5, 10). In these cases, the corresponding deacylated compounds were not taken up by the vacuoles. In this report, we clearly show that neither acylation nor conjugation to glutathione as proposed by Marrs et al. (12) for maize anthocyanins is necessary for vacuolar transport and accumulation of flavonoid-glucosides in barley.

The fact that treatment with NH₄Cl inhibits isovitexin uptake into barley vacuoles strongly suggests that flavonoid-glucoside import may occur via an H⁺/isorvitexin antiport mecha

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REFERENCES

1. Nishida, T., Gatmaitan, Z., Che, M., and Arias, I. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6590–6594
2. Ishikawa, T. (1992) Trends Biochem. Sci. 17, 463–468
3. Deus-Neumann, B., and Zenk, M. H. (1984) Planta 162, 250–260
4. Deus-Neumann, B., and Zenk, M. H. (1986) Planta 167, 44–53
5. Hopp, W., and Seitz, H. U. (1987) Planta 170, 74–85
6. Sandermann, H. (1992) Trends Biochem. Sci. 17, 82–84
7. Werner, C., and Matile, P. (1985) J. Plant Physiol. 118, 237–249
8. Gaillard, C., Dufaud, A., Tommasini, R., Kreuz, K., Amrhein, N., and Martinoia, E. (1994) FEBS Lett. 352, 219–222
9. Stafford, H. A. (1990) Flavonoid Metabolism, CRC Press, Inc., Boca Raton, FL
10. Matern, U., Reichenbach, C., and Heller, W. (1986) Planta 167, 183–189
11. Rataboul, P., Alibert, G., Boller, T., and Boudet, A. M. (1985) Biochim. Biophys. Acta 816, 25–36
12. Marrs, K., Alfenito, M. R., Lloyd, A. M., and Walbot, V. (1995) Nature 375, 397–400
13. Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., and Amrhein, N. (1993) Nature 364, 247–249
14. Li, Z.-S., Zhao, Y., and Rea, P. A. (1995) Plant Physiol. 107, 1257–1268
15. Martinoia, E. (1992) Bot. Acta 105, 223–245
16. Hertensteiner, S., Vogt, E., Hagenbuch, B., Meier, P. J., Amrhein, N., and Martinoia, E. (1993) J. Biol. Chem. 268, 18446–18449
17. Kreuz, K., Tommassini, R., and Martinoia, E. (1996) Plant Physiol. (Bethesda) 111, 349–353
18. Reuber, S., Grieg, D., and Weissenbock, G. (1993) Z. Naturfor. 48c, 749–756
19. Reuber, S., Bornman, J. F., and Weissenbock, G. (1996) Plant Cell Environ. 19, 593–601
20. Seikel, M. K., and Geissman, T. A. (1957) Arch. Biochem. Biophys. 71, 17–30
21. Bowman, E. J., Siebers, A., and Altendorf, K. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7972–7976
22. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
23. Balzi, E., and Goffeau, A. (1994) Biochim. Biophys. Acta 1187, 152–162
24. Getz, H. P., and Klein, M. (1995) Plant Physiol. (Bethesda) 107, 459–467
25. Blume, D. E., Jaworski, J. G., and McClure, J. W. (1979) Planta 140, 199–202