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A herbicide antidote (safener) induces the activity of both the herbicide detoxifying enzyme and of a vacuolar transporter for the detoxified herbicide

Cécile Gaillard[a], Annie Dufaud[a], Roberto Tommasini[b], Klaus Kreuz[c], Nikolaus Amrhein[b], Enrico Martinoia[a,b,*]

[a]*Institut de Biologie de Beau Site Unité Associée CNRS 574, Université de Poitiers, 25 Rue du Faubourg Saint Cyprien, 86 000 Poitiers, France*
[b]*Institute of Plant Sciences, Swiss Federal Institute of Technology, Zürich CH-8092 Zürich, Switzerland*
[c]*Ciba Crop Protetction, Weed Control, Plant Biochemistry, CH-4002 Basel, Switzerland*

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Abstract In plants potentially toxic compounds are ultimately deposited in the large central vacuole. In this report we show that isolated barley mesophyll vacuoles take up the glucoside conjugate of the herbicide derivate [5-hydroxyphenyl]primisulfuron. Transport is stimulated by Mg-ATP and is distinct from that previously described for glutathione conjugates. Treatment of barley with different herbicide antidotes (safeners) revealed that the safener cloquintocet-mexyl doubles the vacuolar transport activities for both the glutathione and glucoside conjugates. Stimulation of the uptake of the metolachlor–glutathione conjugate was the result of an increased uptake velocity whereas the K_m remained unaltered, suggesting that the higher activity was due to a higher expression of the transporter. These results indicate that modulation of vacuolar transport activities are an integral part of the detoxification mechanism of plants.

Key words: Detoxification; Herbicide; Glucoside; Glutathione; Safener; Transport; Vacuole

1. Introduction

Detoxification and elimination of potentially phytotoxic compounds such as microbial toxins and agrochemicals (xenobiotics) present in the environment is a prerequisite for the survival of a plant. Metabolism and detoxification of xenobiotics, which are remarkably similar in plants and animals, can generally be divided into three phases [1,2]. In a first step, which is generally mediated by cytochrome P-450-dependent monooxygenases, a foreign compound may be oxidized, reduced or hydrolyzed to introduce or reveal a functional group. In a second step, which is catalyzed by the respective transferases, the activated xenobiotic is conjugated to a hydrophilic substance such as glutathione, glucuronate, malonate or glucose. In the third step, xenobiotics conjugated to glutathione can be excreted which has been shown to be mediated by a specific ATPase in animals and plants [1,3]. However, while in animals excretion is directed to the extracellular medium, plants sequester the conjugates by compartmentation in the large central vacuole.

Various other mechanisms may further allow the plant to tolerate the presence of xenobiotics. An often very specific effect can be observed in response to the application of so-called safeners (herbicide antidotes). Herbicide safeners are a group of structurally diverse chemicals which increase the tolerance of crops towards specific herbicides. For example, the safener cloquintocet-mexyl confers tolerance to small grain cereals to the herbicide clodinafop-propargyl [4]. The action of safeners has not yet been fully elucidated, but increased activities of glutathione-*S*-transferases, cytochrome P-450-dependent monooxygenases and glucosyl transferases as well as increased GSH contents have been observed in response to safener treatment [5]. The fact that safeners increase GST and glucosyltransferase activities raised the question, whether the vacuolar transport activities for glutathione [3] and glucosyl conjugates (this paper) are part of an integrated defense mechanism and whether they are modulated in response to safener application.

In this report we show that not only glutathione conjugates but also glucosyl conjugates of xenobiotics are actively taken up by vacuoles. The activities of both transporters are modulated in response to a specific safener, indicating that these activities play a central role in the final detoxification of xenobiotics in plants.

2. Materials and methods

Barley (*Hordeum vulgare* L. cv. Baraka) was grown on vermiculite in a controlled environment under the following conditions: 16 h light (14 $W \cdot m^{-2}$, Sylvania tubes F65 W gro-lux) at 22 ± 1°C with a relative humidity of 65 ± 5%. The plants were watered daily with Hoagland's solution. Safeners were dissolved in ethanol (10 mM stock solution) and applied at a concentration of 10 μM in Hoagland's solution at day 1, 4 and 7. Salicylic acid and *p*-hydroxybenzoic acid were buffered to pH 7.0 with 10 mM HEPES-KOH and applied at a concentration of 6 mM at day 5, 6 and 7. The effect of salicylic acid was calculated as the difference between the transport activites of vacuoles isolated from plants treated with salicylic acid and *p*-hydroxybenzoic acid, respectively.

All chemicals and solvents were of highest purity available and were mainly supplied by Fluka and Sigma. [^{14}C]Metolachlor-GS (0.2 MBq/μmol) was synthesized as described by Fuerst and Gronwald [6]. Hydroxyprimisulfuron-[^{14}C]glucoside (0.37 MBq/μmol) was synthesized enzymatically from [5-hydroxyphenyl]primisulfuron (for structure see [7]) and UDP-[U-^{14}C]glucose using partially purified glucosyl transferase from maize (Kreuz, unpublished results). The radiochemical purity of the hydroxyprimisulfuron-[^{14}C]glucoside was 97% as judged by thin-layer chromatography.

*Corresponding author. Fax: (33) 49 55 93 74.

Abbreviations: GST, glutathione-*S*-transferase; HPS-G, hydroxyprimisulfuron-glucoside; MOC-GS, metolachlor-glutathione.

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Glutathione-*S*-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate was assayed essentially according to [8].

Primary leaves of 8-day-old barley plants were harvested at the beginning of the light period. Barley mesophyll protoplasts and vacuoles were prepared as described by Rentsch and Martinoia [9].

Uptake of [^{14}C]metolachlor, hydroxyprimisulfuron-[^{14}C]glucoside and [^{14}C]sucrose into vacuoles was measured essentially as described [3]. For each condition and time point, five polyethylene microcentrifugation tubes (400 μl capacity) were prepared as follows: 70 μl of incubation medium containing 30% (v/v) Percoll, 400 mM sorbitol, 30 mM K-gluconate, 0.12% bovine serum albumin (BSA), 0.9 kBq of [^{14}C]metolachlor, 1.85 kBq hydroxyprimisulfuron-[^{14}C]glucoside or 1.85 kBq [^{14}C]sucrose, 3.7 kBq ^3H_2O and solutes as indicated in the figures and tables were placed in the bottom of the tube. Uptake was started by the addition of 30 μl vacuole suspension. The samples were rapidly overlayered 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 s. Radioactivity was determined in 50 μl of the upper aqueous phase containing the hypotonically shocked vacuoles. Unless stated otherwise, uptake rates were calculated by subtracting the radioactivity taken up after 2 min from the 12 min ([^{14}C]metolachlor-GS) or 20 min (hydroxyprimisulfuron-[^{14}C]glucoside, [^{14}C]sucrose) values. ^3H_2O equilibrates rapidly between the medium and the vacuolar space and can thus be used to quantify the number of vacuoles (10^7 vacuoles correspond to a volume of 160 μl).

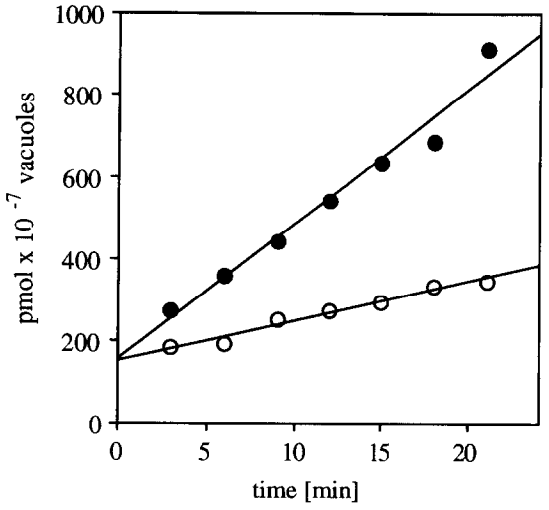

Fig. 1. Time-dependent uptake of hydroxyprimisulfuron-[^{14}C]glucoside into isolated barley mesophyll vacuoles in absence (○) or presence (●) of 3 mM Mg-ATP. Isolated vacuoles were incubated in the presence of 30 μM hydroxyprimisulfuron-[^{14}C]glucoside for different times as described in section 2. Each point is the mean of two values.

3. Results and discussion

We have recently demonstrated that uptake of herbicides conjugated to glutathione into vacuoles is mediated by a primary active transporter which has properties very similar to those of the glutathione-*S*-conjugate ATPase described for the canalicular membrane of liver [3]. This system for plant vacuoles appears to be ubiquitous, since we found similar acitivities in carrot tap root vacuoles and lutoids of *Hevea brasiliensis* (results not shown). Glucose conjugates of xenobiotics have also been shown to be deposited in the large central vacuole of plants [10]; however, uptake of these compounds into isolated vacuoles has previously not been demonstrated. As shown in Fig. 1, glucosyl conjugates of herbicides are also taken up by isolated vacuoles. Uptake of hydroxyprimisulfuron-[^{14}C]glucoside is linear for at least 20 min and is stimulated three to five times by Mg-ATP. A similar, ATP-dependent uptake activity has been shown to occur in isolated barley vacuoles for a biological glucoside, esculine [11]. The uptake rate for hydroxyprimisulfuron-[^{14}C]glucoside is about 30-fold lower than that observed for MOC-GS at the same concentration. This uptake system is distinct from the glutathione conjugate ATPase. Esculine inhibits the uptake of the herbicide conjugated to the glucose moiety, while no inhibition could be observed for the glutathione conjugate (Table 1). This observation suggests that glucose conjugates of biological substances as well as of xenobiotics may cross the tonoplast by a common transport mechanism.

Table 1
Inhibition of [^{14}C]-metolachlor-glutathione and hydroxyprimisulfuron-[^{14}C]glucoside uptake by esculine

	Uptake (% of control)	
	MOC-GS	HPS-G
Experiment 1	95 ± 5	59 ± 12
Experiment 2	101 ± 6	61 ± 10

Barley vacuoles where incubated with 30 μM of the respective herbicide conjugate and 3 mM Mg-ATP, in absence or presence of 1 mM esculine. Uptake rates (pmol · 10^{-7} vacuoles · min^{-1}) for the control values were 608 and 582 (MOC-GS) and 19.2 and 16.8 (HPS-G). For each experiment means of five replicates ± S.D. The higher S.D. for HPS-G is due to the lower uptake rate.

Herbicide safeners enhance the tolerance of a crop against a specific herbicide by causing a concerted increase in the activities of GST, glucosyl transferases and cytochrome P-450-dependent monooxygenases, as well as in the GSH content [5]. Our results demonstrate that vacuolar transporters responsible for the uptake of glutathione and glucosyl conjugates are also induced after treatment of barley plants with such a substance. Cloquintocet-mexyl, a safener which enhances the tolerance of small grain cereals to the herbicide clodinafop-propargyl [4], doubled the rate of uptake of both conjugates, while sucrose uptake was slightly inhibited by the safener treatment (Table 2). Induction of hydroxyprimisulfuron-glucoside uptake was always at least 20% higher than that of MOC-GS. In contrast, under the conditions employed, the safeners naphthalic anhydride and benoxacor which are known to increase tolerance of maize towards the herbicides EPTC and metolachlor, respectively, had almost no effect on the transport activity of the herbicide conjugates. As shown in Table 2, GST activity was found to be significantly induced in leaf extracts from plants treated with cloquintocet-mexyl, whereas naphthalic anhydride and benoxacor only marginally influenced GST activity. As expected, both constitutive and induced GST activities were fairly low in 8-day-old barley plants; a rapid decline in GST activity after seedling emergence has been reported in maize [12]. Much less is known on the induction by a safener of the glucosyl transferase activity, and it would be interesting to compare such data with those for GST. In contrast, safeners which are known to confer resistance to maize, but not in barley, against the herbicides EPTC and metolachlor had no effect on the induction of vacuolar transporters in barley. Expression of various tolerance mechanisms against xenobiotics appears therefore to be triggered by a common signal. As shown in Fig. 2, treatment of barley plants with the safener

Table 2
Uptake of MOC-GS, HPS-glucose and sucrose and measurement of GST activity in response to safener and salicylic acid treatment

Treatment	Uptake (% of control)			
	MOC-GS (*n*)	HPS-glucose (*n*)	Sucrose (*n*)	GST
Control	100	100	100	100
Naphthalic anhydride	102 ± 12 (3)	98 (2)	n.d.	122 ± 10
Benoxacor	110 ± 9 (3)	111 (2)	n.d.	123 ± 12
Cloquintocet-mexyl	189 ± 30 (4)	216 ± 14 (3)	78 (2)	151 ± 4
Salicylic acid	109 ± 5 (3)	132 ± 14 (3)	n.d.	n.d.

Isolated barley mesophyll vacuoles from barley treated with 10 mM safeners or 6 mM salicylic acid (for details see section 2) were incubated in the presence of 30 mM [^{14}C]metolachlor, 25 mM hydroxyprimisulfuron-[^{14}C]glucoside or 1 mM [^{14}C]sucose. Uptake rates for the control values were 512 ± 84 (MOC-GS), 13.9 ± 2.1 (HPS-G), 1400 (sucrose) pmol × 10^{-7}vacuoles·min^{-1}; GST activity in the control was 86 ± 11 nmol·mg^{-1} protein·min^{-1}. Means of *n* experiments ± S.D., each with five replicates. GST activity was measured using. 1-chloro-2,4-dinitrobenzene as substrate (for details see section 2).

cloquintocet-mexyl only affected the maximal velocity (V_{max}, increase from 2.4 to 4.9 nmol × 10^{-7} vacuoles·min^{-1} in this experiment) but not the K_m of metolachlor-GS transport (115 μM). The fact that only a change in V_{max} but not in K_m was observed after treatment with the safener suggests that the enhanced transport activity is due to an enhanced incorporation of the transporters into the vacuolar membrane under these conditions.

Salicylic acid is known to confer systemic resistance against pathogens in many plants [13,14]. Various pathogen-related proteins (PRP) are synthesized in response to salicylic acid treatment and one of these proteins has recently been identified as a GST [15]. We were therefore interested whether the conjugate transport activities of barley vacuoles increase in response to salicylic acid treatment. In contrast to safener treatment, the activity of the glutathione conjugate ATPase increased only by approximately 10% as a result of salicylic acid treatment. A more pronounced induction (about 30%) was observed for the uptake of hydroxyprimisulfuron-glucoside. But the activity resulting from safener treatment was distinctly higher. No change in transport activity could be observed when protoplasts were incubated in the presence of cloquintocet-mexyl or salicylic acid for three hours prior to vacuole isolation. The fact that hydroxyprimisulfuron-glucoside uptake is enhanced stronger than the uptake of glutathione conjugates may be due to the fact that in vivo salicylic acid is conjugated to glucose. However, barley synthesizes only negligible amounts of extracellular PRP in response to salicylic acid (Schweitzer, personal communication) and the activities of vacuolar transporters may thus be enhanced much stronger in other plants in response to salicylic acid treatment.

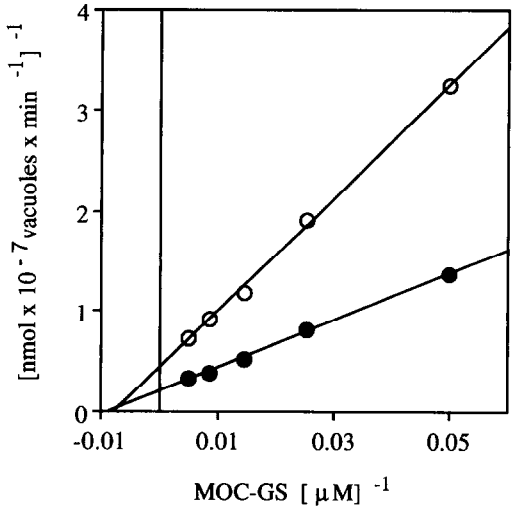

Fig. 2. Lineweaver-Burk plots of the transport of [^{14}C]metolachlor-glutathione into barley mesophyll vacuoles as a function of its concentration. Vacuoles from control (○) and safener (●) treated plants were incubated for 12 min in the presence of 18.5 kBq·ml^{-1} metolachlor-GS and various concentrations of unlabeled metolachlor-GS.

4. Conclusions

A signal may induce a concerted increase not only of the GST and P-450 monooxygenases activities but also of the vacuolar transport activities for xenobiotics conjugated either to glutathione or glucose. This observation suggests that excretion of conjugated xenobiotics into the vacuole is an integral part of the detoxification mechanism of a plant.

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