RESEARCH PAPER

Use of D-glucose–fenpiclonil conjugate as a potent and specific inhibitor of sucrose carriers

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Abstract

Until now, specific inhibitors of sucrose carriers were not available. This led us to study the properties of the recently synthesized D-glucose–fenpiclonil conjugate (D-GFC). This large amphiphilic glucoside exhibited an extremely low phloem systemicity in contrast to L-amino acid–fenpiclonil conjugates. Using Ricinus seedlings, the effect of D-GFC on 0.5 mM [14C]sucrose (Suc), 3-O-[3H]methylglucose, and [3H]glutamine uptake by cotyledon tissues was compared with that of p-chloromercuribenzene-sulfonic acid (PCMS). D-GFC dramatically inhibited H+-Suc symport at the same concentrations as PCMS (0.5 and 1 mM), but in contrast to the thiol reagent, it did not affect 3-O-methylglucose and glutamine transport, nor the acidification of the incubation medium by cotyledon tissues. Similarly, 0.5 mM D-GFC inhibited active Suc uptake by Vicia faba leaf tissues and by Saccharomyces cerevisiae cells transformed with AtSUC2, a gene involved in Suc phloem loading in Arabidopsis, by approximately 80%. The data indicated that D-GFC was a potent inhibitor of Suc uptake from the endosperm and of Suc phloem loading. It is the first chemical known to exhibit such specificity, at least in Ricinus, and this property permitted the quantification of the two routes involved in phloem loading of endogenous sugars after endosperm removal.

Key words: Apoplastic loaders, Arabidopsis AtSUC2, D-glucose–fenpiclonil conjugate, PCMS, phloem loading, Ricinus seedlings, specific inhibition of sucrose carriers, sucrose uptake from endosperm, Vicia faba.

Introduction

The non-permeant or poorly permeant sulphydryl reagent p-chloromercuribenzenesulfonic acid (PCMS) has been successfully used in phloem biology, first to demonstrate that sucrose accumulates in the phloem symplasm from the vein apoplasma through Suc carriers as in Beta vulgaris and Vicia faba (Giaquinta, 1976; Delrot et al., 1980; Giaquinta, 1983), and then to identify apoplastic and symplasmic loaders (van Bel et al., 1992; Turgeon and Medville, 2004; Turgeon and Ayre, 2005). PCMS dramatically inhibits the activity of Suc carriers but because it reacts with cysteine residues of many other plasma membrane (PM) intrinsic proteins, it also affects the transport of other solutes, as demonstrated in...
Ricinus communis (Williams et al., 1996; Rocher et al., 2009; Tamas and Davies, 2016). The effect of PCMBS on the PM H+-ATPase varies with tissues. For instance, it does not significantly affect the proton pumping activity nor the transmembrane potential difference in mature broad bean leaf tissues (Delrot et al., 1980; Bourquin et al., 1990), but it inhibits proton pumping by microsomal vesicles and acidification of the incubation medium by intact cotyledons of Ricinus communis seedlings (Williams and Hall, 1987; Williams et al., 1990).

The Ricinus seedling has been successfully used as a model plant to study the composition of the phloem sap (Kriedemann and Bevers, 1967; Schobert and Komor, 1989; Vreugdenhil and Koot-Gronsveld, 1989; Gerendas and Schurr, 1999; Kallarackal et al., 2012), the mechanisms of nutrient uptake by cotyledon tissues and phloem loading (Komor et al., 1977; Komor et al., 1980; Robinson and Bevers, 1981b; Marvier et al., 1998; Williams et al., 1996), as well as the long-distance transport of sugars (Kallarackal et al., 1989; Metzler et al., 1994; Verscht et al., 1998; Kallarackal et al., 2012). As in most plant species (Liu et al., 2012), sucrose (Suc) is the major sugar of the Ricinus phloem sap, with concentrations of approximately 300 mM in intact seedlings (Kallarackal et al., 1989; Verscht et al., 1998). Glucose and fructose have much lower concentrations (approximately 2 and 0.6 mM, respectively) (Kallarackal and Komor, 1989). However, the phloem of Ricinus seedlings exhibits the peculiarity of loading exogenous glucose. When cotyledons are dipped in solutions containing from 25 to 200 mM glucose, the same concentrations as in the incubation solutions are found in the phloem sap after 2 h of incubation (Kallarackal and Komor, 1989).

The Ricinus seedling has also been used to study the phloem mobility of xenobiotic conjugates, i.e. compounds that associate an agrochemical and an α-amino acid (Dufaud et al., 1994; Chollet et al., 1997; Delétage-Grandon et al., 2001; Wu et al., 2016; Xie et al., 2016) or a monosaccharide (Yang et al., 2011; Wu et al., 2012; Yuan et al., 2013) in their structure. This vectorization strategy has been developed to evaluate the ability of PM carriers to translocate large and chlorinated xenobiotics (Delétage-Grandon et al., 2001; Yang et al., 2011; Wu et al., 2012; Yuan et al., 2013). We have recently synthesized two types of conjugate of fenpiclonil, a contact fungicide from the phenylpyrrole family used as a model molecule, namely, a D-glucose conjugate and an L- and a D-glutamic acid conjugate (Wu et al., 2016). These compounds, which violate both Lipinski’s (Lipinski et al., 2012) and Veber’s (Veber et al., 2002) rules, were predicted to have very low membrane permeability. Nevertheless, they were found in the phloem sap. Systemic tests using the Ricinus model indicated that these large chlorinated conjugates exhibited dramatic differences in their ability to move in the phloem. When cotyledons were dipped in an incubation solution buffered at pH 5.0, the concentrations of the

Fig. 1. Two- and three-dimensional structure of D-GFC obtained using Chem3D Pro, energy minimization with the MM2 method. Atoms are denoted by spheres in the following colours: carbon in pale grey, hydrogen in light blue, chlorine in green, oxygen in red, and nitrogen in blue. For this compound, $M_r=551.38$ and $K_{ow}=0.71$ (computed with ACD/Labs Percepta 2015 release).

Fig. 2. Phloem sap exudation from Ricinus seedlings. (A) pH time course variation of the Ricinus phloem sap. After preincubation for 60 min in a standard medium (pH 5.0), the hypocotyls were severed, and then the phloem sap was collected each hour from the upper part for pH measurements. Each point is the mean±95% CI of three sets of five plants. (B) Effect of 1 M CaCl$_2$ on sap exudation in the presence of D-GFC. After preincubation for 30 min in a standard medium (pH 5.0), the cotyledons were dipped in the same solution containing 0.5 mM D-GFC for 30 min. Then the hypocotyls were severed and the phloem sap was collected each hour from the upper part for volume measurements. In the treated sets, 1 M CaCl$_2$ solution was applied to the hypocotyl section just after the second sap collection (arrowhead). Each point is the mean of 10 plants±95% CI.
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D-glucose conjugate and the D-glutamic acid conjugate in the phloem sap were 20 and 5 times lower than that of the L-glutamic acid conjugate, respectively. The phloem systemicity of the fenpiclonil glucoside was even 30–45 times lower than that of the most recent L-amino acid–fenpiclonil conjugates synthesized (Marhadour et al., 2017). Depending on their structure, natural glucosides exhibit different abilities to move in the phloem. For instance, glucosinolates are translocated long distance within the plant (Chen et al., 2001; Turgeon and Wolf, 2009), and two members of the nitrate/peptide transporter family (GTR1 and GTR2) are involved in the phloem loading of these defence compounds (Nour-Eldin et al., 2012). Small and hydrophilic glucosides such as salicin (Mr=286.28; log P=-1.48) and arbutin (Mr=272.25; log P=-1.14) are translocated by AtSUC2 expressed in Xenopus laevis oocytes (Chandran et al., 2003). By contrast, the presence of phlorizin in the phloem sap has not been reported until now. This glucoside of phloretin is a non-transported competitive inhibitor of Na+-glucose cotransporters in animal cells (Toggenburger et al., 1982; Hummel et al., 2011). In plants, phlorizin is recognized by hexose and Suc carrier systems but more efficiently inhibits Suc phloem loading than hexose uptake in broad bean leaves (Lemoine and Delrot, 1987). Due to the glycosyl hydroxyls (Hitz et al., 1986; Delrot et al., 1991), D-GFC may be recognized by Suc carriers but the size (Mr=551.38) and the amphiphilic structure (Fig. 1) of the compound are completely inappropriate for translocation considering the molecular structural requirements suitable for Suc carrier activity (Hitz et al., 1986; Delrot et al., 1991; Chandran et al., 2003). Therefore, we hypothesized that D-GFC would affect sugar translocation systems. We use Ricinus as our model to test this hypothesis because it can load in the phloem not only endogenous Suc but also exogenous hexoses as mentioned above.

The purpose of this work was initially to compare the effect of D-GFC and PCMBS on Suc, 3-O-methylglucose...
(3-O-MeG) and glutamine (Gln) uptake and phloem transport in *Ricinus* seedlings. The results allowed a quantitative study of the contribution of the two pathways involved in phloem loading after endosperm removal and led us to extend the investigation to other biological models.

**Materials and methods**

**Plant material**

Castor bean seeds (*Ricinus communis* L. cv Sanguineus) were grown as previously described (Delétage-Grandon *et al.*, 2001). After 6 days of growth in vermiculite, seedlings of average size were selected for the experiments.

Broad bean (*Vicia faba* cv Aguadulce) plants were grown on vermiculite and watered daily with a nutrient solution as already described (Lemoine *et al.*, 1984). The experiments were performed on plants possessing five mature bifoliate leaves.

*Saccharomyces cerevisiae* strain RS453 cells were grown and transformed as described in Henry *et al.* (2011).

**Chemicals**

We have previously described the detailed synthesis of the D-glucose–fenpiclonil conjugate (D-GFC; Fig. 1) (Wu *et al.*, 2016). This conjugate was obtained using click chemistry, by coupling fenpiclonil, a fungicide from the phenylpyrrole family, to D-glucose via a spacer group including a 1,2,3-triazole ring.

PCMBS was purchased from Toronto Research Chemicals Inc., 3-0-[3H]MeG (60 Ci mmol−1) was purchased from Isobio and [3H]Gln (50.1 Ci mmol−1) and [U-14C]Suc (435 mCi mmol−1) were purchased from PerkinElmer SAS.

**Uptake and exudation experiments with Ricinus seedlings**

The cotyledons were preincubated in the standard solution buffered at pH 5.0 for 30 min and then incubated in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC. Thirty minutes later, 3-O-[3H]MeG and unlabelled 3-O-MeG were added to the solution to get 0.5 mM final concentration (specific activity: 0.30 mCi mmol−1; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of 3-O-MeG (and metabolites) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. (A, B) t=1h, P=0.009; t=2h, P=0.023; t=3h, P=0.052; t=4h, P=0.143; t=5h, P=0.075. (C, D) no statistically significant difference was noted. For box plots, n=10.

![Fig. 4. Effect of D-GFC on 3-O-MeG uptake in the *Ricinus* model. Cotyledons were preincubated in a standard solution buffered at pH 5.0 for 30 min and then incubated in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC. Thirty minutes later, 3-O-[3H]MeG and unlabelled 3-O-MeG were added to the solution to get 0.5 mM final concentration (specific activity: 0.30 mCi mmol−1; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of 3-O-MeG (and metabolites) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. (A, B) t=1h, P=0.009; t=2h, P=0.023; t=3h, P=0.052; t=4h, P=0.143; t=5h, P=0.075. (C, D) no statistically significant difference was noted. For box plots, n=10.](https://academic.oup.com/jxb/article-abstract/68/20/5599/4569173)
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phloem exudation of the exogenous solutes. The phloem sap was collected from the upper part of the hypocotyl for 5 h and was stored at −20 °C until analysis. Phloem sap was added to 4 ml scintillation cocktail (EcoLite, ICN Biomedicals). Radioactivity was measured with a liquid scintillation analyser (Tri-Carb 2910TR, PerkinElmer).

At the end of the exudation period, the cotyledons were rinsed with the buffer solution (3 × 2 min), wiped off with filter paper and weighed. Then, the cotyledons were digested overnight at 60 °C in a mixture of perchloric acid (65%; 0.56 ml), hydrogen peroxide (33%; 0 27 ml), and Triton X-100 (1 g l⁻¹; 0.17 ml). Radioactivity measurements were conducted on each seedling separately.

Analysis of endogenous sucrose, glucose, and fructose in Ricinus phloem sap

The Ricinus cotyledons were incubated in buffer solution (from pH 5.0 to 8.0) containing 0.25 mM MgCl₂ and 0.5 mM CaCl₂. The buffer used was 20 mM MES (pH 5.0 and 6.0) or 20 mM HEPES (pH 7.0 and 8.0) (Rocher et al., 2006). The phloem sap was collected from the upper part of the Ricinus seedlings according to the methods already described (Kallarackal et al., 1989). After removing the endosperm, the cotyledons of intact seedlings were preincubated for 30 min in the buffer solution. Then the cotyledons were incubated in the same buffer solution with or without 0.5 mM D-GFC. Thirty minutes later, the hypocotyl was cut in the hook region and the collected phloem sap was stored at −20 °C until analysis.

The endogenous sugars glucose, fructose, and Suc in the phloem sap were determined enzymatically using previously described methods (Orlich and Komor, 1992). A Suc/D-fructose/D-glucose assay kit (K-SUFRG; Megazyme, Ltd, Bray, Ireland) was used following the manufacturer’s instructions. To measure the Suc concentration, the phloem sap was diluted 100-fold with pure water. All measurements were performed by using 1/4 of the amounts of the reagents recommended by the manufacturer.

pH transients in the incubation solution of Ricinus seedlings

The measurement of pH transients in the medium using Ricinus cotyledons was similar to that described previously (Komor et al., 1977; Hutchings, 1978; Robinson and Beevers, 1981b). The cotyledon still
attached to the seedling was incubated in a solution (10 ml) containing 0.25 mM MgCl₂ and 0.5 mM CaCl₂. The solution was stirred continuously and the pH of the solution was monitored every 30 s with a microelectrode and a pH meter (S220 SevenCompact pH/Ion Meter, Mettler Toledo). The pH of the solution bathing the cotyledons decreased to pH 4.6–4.9 after 30 min then stabilized between 4.8 and 5.0. Small aliquots of concentrated solutions of D-GFC or PCMBs did not affect the transmembrane proton gradient in intact cotyledons (Rocher et al., 2009). Furthermore, it was confirmed that 0.5 mM D-GFC exhibited no statistically significant effect on Gln uptake. The Mann–Whitney test was used to assess statistically significant differences between controls and treated sets at the 5% probability level. For 3-O-MeG, P = 0.00014; for Gln, P = 0.0014. For box plots, n = 15 (3-O-MeG) or n = 12 (Gln).

### Uptake experiments in yeast

The AtsUC2 coding region in the plasmid pDONR207 coding region was a generous gift from Dr F. Vilaine (Institut Jean Pierre Bourgin, Versailles, France). The coding region was cloned by recombination into plasmid pDR-R1-R2-HIS3 (Cagnac et al., 2007) derived from pDR 192. The plasmid containing AtsUC2 and the empty plasmid were inserted into Saccharomyces cerevisiae RS453 and Suc uptake experiments were run as described in Henry et al. (2011). Briefly, yeast cells were grown to early logarithmic phase in YNB medium supplemented with 2% glucose. Cells were washed and resuspended with 50 mM MES buffer (pH 4.5) to reach a final OD₆₀₀ₐₜₜ value of 0.5. Aliquots (100 µl) of cell suspension were added to 100 µl of a solution containing 50 mM MES (pH 4.5) and a mixture of unlabelled and ³⁵C-labelled Suc (concentration: 1 mM; specific activity: 0.50 mCi mmol⁻¹) at 28 °C for 5 min. The final sucrose concentration in the medium was therefore 0.5 mM.

The reactions were stopped by adding 8 ml of cold water and immediate filtration on glass microfiber filters (25 mm, Fisher Bioblock, Illkirch, France). This step was repeated once. Radioactivity incorporated into cells collected on filters was evaluated using a liquid scintillation counter.

### Results and discussion

**Effect of the D-glucose–fenpiclonil conjugate on the uptake and phloem transport of exogenous Suc, 3-O-MeG, and Gln in Ricinus seedlings in comparison with PCMBs**

The two nutrients and the sugar analogue were selected because of their high concentrations in the phloem sap and/or their poor metabolism. Suc is slowly metabolized in Ricinus cotyledons (Kriedemann and Bevers, 1967; Komor et al., 1977) and represents 98–99% of the total sugar in the phloem sap (Kallarackal and Komor, 1989). The mobile analogue 3-O-MeG is slowly phosphorylated in plant tissues (Cortès et al., 2003). Chromatography analyses suggest the absence of metabolic transformation of 3-O-MeG in the Ricinus phloem exudate under short-time experiments (2 h) (Kallarackal and Komor, 1989). Gln is the major amino acid found in the Ricinus phloem sap, comprising 30–40% of the total amino acids (Robinson and Bevers, 1981a; Schobert and Komor, 1989) and dramatically accumulates in the phloem sap from the apoplast between the endosperm and the cotyledons (endogenous Gln) or from an incubation solution (exogenous Gln) (Schobert and Komor, 1989). The cotyledon tissues acidified the non-buffered solution to pH values of 4.8–5.0 as mentioned above. Therefore the experiments were conducted using incubation solutions buffered at pH 5.0.

Contrary to species of the Cucurbitaceae (Zhang et al., 2012; Zimmermann et al., 2013), after Ricinus hypocotyls were cut under our experimental conditions, an immediate and strong efflux of a mixture of phloem and xylem sap did not occur from the apical side of the cut. The concentration of Suc, which was approximately 300 mM in intact seedlings,
D-Glucose fenpiclonil is a new sucrose carrier inhibitor decreased to approximately 100 mM 2 h after endosperm removal (Kallarackal and Komor, 1989) and to approximately 160 mM in our plant material. The concentrations of glucose and fructose were very low, approximately 2 and 0.7 mM, respectively. The concentrations of these three sugars remained stable for at least 5 h as detailed below. The pH of the sap remained almost unchanged (7.9–7.6) (Fig. 2A). After addition of 1 M CaCl₂, which induced a biphasic occlusion of sieve tubes (Furch et al., 2010), no fluid in measurable quantities was released from the apical cut until the end of the experiment. The result was the same in the presence of D-GFC (Fig. 2B). All the data suggest that the phloem sap could be collected for at least 5 h after the first hour of preincubation, i.e. 6 h after endosperm removal.

Considering that a relatively small amino acid conjugate (L-Lys–2,4D; \( M_r = 349.21 \)) at 2.5 mM concentration was, in acidic conditions, an efficient inhibitor of uptake and phloem loading of various amino acids at a 1 mM concentration (Chollet et al., 1997), we speculated that the large amphiphilic conjugate D-GFC could be used at low concentrations to affect the active uptake mediated by sugar carriers under their protonated form. Therefore, to compare the effects of D-GFC on uptake and phloem transport of the two sugars and the amino acid, the conjugate was used at 0.5 or 1 mM and exogenous Suc, 3-O-MeG, and Gln were used at 0.5 mM concentration in the incubation medium. Under
these experimental conditions, D-GFC acted as a potent inhibitor of Suc uptake, especially Suc phloem transport. In a control set, exogenous Suc concentration in the phloem sap increased over time to reach a concentration factor (CF; concentration in the phloem sap/concentration in the incubation solution) of approximately 25 at the end of the experiment (Fig. 3A). In the treated set, the concentration of exogenous Suc in sieve tubes decreased by approximately 70% regardless of the time of sap collection (Fig. 3B). The amount of exogenous Suc and metabolites (in Suc equivalent) in cotyledon tissues collected at the end of the experiment (5 h) was reduced by 55% in the treated set (Fig. 3C, D). A slightly higher inhibition (65%; 0.96 and 0.34 µmol exogenous Suc g⁻¹ fresh weight of Ricinus cotyledons in control and treated sets, respectively) occurred in a short-time experiment (1 h). As discussed below, Suc uptake by Ricinus cotyledons is also reduced by PCMBS (Orlich et al., 1998). By contrast, D-GFC did not negatively affect the concentration of 3-O-MeG in the phloem sap, regardless of the time of phloem collection (Fig. 4A, B). This concentration increased over time to reach a CF of approximately 2 at the end of the experiment in both sets and was weakly but nevertheless significantly higher (Mann–Whitney test) in the treated set than in the control at times 1, 2, and 3 h. The amounts of 3-O-MeG and metabolites in cotyledon tissues were the same in both sets at 5 h (Fig. 4C, D). Similarly, the conjugate did not induce changes in the time course of accumulation of exogenous Gln and metabolites (in Gln equivalent) in the phloem sap. In both sets, their concentration plateaued during the last 2–3 h of the experiments to reach a CF of approximately 6 (Fig. 5A, B). In addition, the amounts of exogenous Gln and metabolites in cotyledon tissues were the same in control and treated sets (Fig. 5C, D). By contrast, 0.5 mM PCMBS reduced the uptake of 3-O-MeG in cotyledon tissues by 47% under short-time experiments (Fig. 6A, B). These experimental conditions were necessary because this thiol reagent progressively affects transmembrane proton gradients in Ricinus as mentioned below. At the same concentration, PCMBS moderately
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Affected (approximately 25% inhibition) Gln uptake by tissues (Fig. 6C, D) but the inhibition was higher (≥50%) using plasma membrane vesicles from Ricinus cotyledons (Williams et al., 1992).

Considering these results, complementary experiments were performed to get additional data on D-GFC specificity and some insights on concentration and pH dependence, as well as post-treatment duration of Suc transport inhibition induced by the chemical. Although 0.5 mM D-GFC led to a dramatic inhibition of Suc phloem loading in Ricinus, the chemical was used at 1 mM in the following experiments. Under this experimental condition, D-GFC did not affect the time course of incubation medium acidification by cotyledon tissues (Fig. 7A, B) contrary to PCMBS (Fig. 7C). Therefore this result provided evidence that D-GFC did not alter the activity of the plasma membrane H⁺-ATPase via toxic effects on some cell functions, even for long-term experiments (several hours). In addition, 1 mM PCMBS quickly reduced the volume of phloem sap by 75% (Orlich et al., 1998; Fig. 4B), while D-GFC had a less dramatic effect (from 32 to 42% inhibition) at the same concentration and a weak effect at 0.5 mM (Fig. 8A, B).

Increasing conjugate concentration from 0.5 to 1 mM in the incubation medium buffered at pH 5.0 led to an

**Table 1.** Effect of 1 mM D-GFC on 0.5 mM Suc uptake (µmol g⁻¹ fresh material) by Ricinus cotyledon tissues at pH values of 5.0 and 8.0

Cotyledons were preincubated in a standard solution buffered at pH 5.0 or pH 8.0 for 30 min and then incubated in the same solution containing 0 mM (Control) or 1 mM (Treated set) D-GFC (10 µl per plant). Thirty minutes later, [¹⁴C]Suc and unlabelled Suc were added to the solution to get 0.5 mM final concentration (specific activity: 0.04 mCi mmol⁻¹). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h (see Fig. 9). At the end of experiment, the amount of exogenous Suc and metabolites (in Suc equivalent) in cotyledon tissues was determined by liquid scintillation counting. The data are means±95% CI, n=10.

| pH 5.0 | Control | Treated set | Inhibition (%) |
|--------|---------|-------------|---------------|
| 1.98 ± 0.23 | 0.59 ± 0.08 | −70 |

| pH 8.0 | Control | Treated set | Inhibition (%) |
|--------|---------|-------------|---------------|
| 0.97 ± 0.10 | 0.79 ± 0.05 | −19 |

![Fig. 9.](https://academic.oup.com/jxb/article-abstract/68/20/5599/4569173)
accentuated decrease of exogenous Suc concentration in the phloem sap (80–85% vs 70%) regardless of the time of sap collection (compare Fig. 3A, B and Fig. 9A, B). The same was observed for the amounts of exogenous Suc and metabolites in cotyledon tissues collected at the end of experiments (approximately 70% vs 55%) (compare Fig. 3C, D and Table 1). The uptake and phloem transport of exogenous Suc were either markedly dependent (control sets) or independent (treated sets) of the incubation medium pH. In control sets, the concentration of exogenous Suc in the sap and its amount in cotyledon tissues at 5 h were both reduced by approximately 50% from pH 5.0 to 8.0 (Fig. 9A, C, E; Table 1). By contrast, in the treated set, the low exogenous Suc contents in these two compartments were unaffected (Fig. 9B, D, F) or were weakly affected (Table 1) by the pH change, suggesting that 1 mM D-GFC reduced to nothing, or almost nothing, the pH-dependent component of the active Suc transport, i.e. the symport H+–Suc. This well-known mechanism described several decades ago (Komor, 1977; Komor et al., 1977; Hutchings, 1978; Malek and Baker, 1978; Delrot and Bonnemain, 1979, 1981) was notably supported by an alkalinization of the incubation medium due to concomitant influxes of protons and Suc in cotyledon tissues. At 1 mM, D-GFC completely abolished the transient alkalinization induced by these influxes after the addition of 20 mM Suc (final concentration) in the incubation solution (Fig. 7D, E). The inhibition of the active component of Suc transport was thoroughly removed after a short washing (3 × 2 min) of cotyledon tissues in the standard medium. Besides previous incubation of tissues for 1 h in a solution containing 1 mM conjugate (treated set) before washing, the time course enrichment of exogenous Suc in the phloem sap (Fig. 10A, B) and Suc uptake by cotyledon tissues (Fig. 10C, D) were similar in the control and treated sets.

**Fig. 10.** Reversibility of the inhibitory effect of D-GFC on Suc uptake in the Ricinus model. Cotyledons were incubated in a standard buffered solution at pH 5.0 for 30 min and then in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC for 60 min. Cotyledons were then washed 3 × 2 min with the standard buffer solution and were incubated (control and treated set) in the standard solution containing 0.5 mM [14C]Suc and unlabelled Suc (final concentration, specific activity: 0.04 mCi mmol⁻¹; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of [14C]Suc (and labelled metabolites) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. (A, B) For each time, no significant differences were noted; (C, D) no significant difference was noted. For box plots, n=10.
While PCMBS forms covalent bonds with protein cysteine residues, D-GFC acts as a reversible inhibitor like the natural glucoside phlorizin (Bush, 1993). The thiol reagent and D-GFC exhibit similarities and differences. The most astonishing similarity is that the conjugate (described here) and PCMBS (Orlich et al., 1998) inhibit Suc transport in *Ricinus* by 80–90% at the same concentration (1 mM). The most striking difference is the specificity of D-GFC, which does not affect Gln or 3-O-MeG transport in cotyledon tissues, contrary to PCMBS. In addition, the xenobiotic glucoside does not change the time course acidification of the incubation medium by cotyledon tissues while the sulfhydryl reagent alters this process (Williams and Hall, 1987; Williams et al., 1990; our data). It has been well known for decades, first by using plant tissues and then plasma membrane vesicles or complemented yeast mutants, that PCMBS inhibits not only Suc carriers (Delrot et al., 1980; Bush, 1993; Lemoine, 2000; Barth et al., 2003; Sauer, 2007) and oligopeptide carriers (Jamai et al., 1994; Rentsch et al., 1995), but also amino acid transporter systems to various degrees (from 5 to 75%) (Servaites et al., 1979; Despeghel and Delrot, 1983; M’Batchi and Delrot, 1984; Montamat et al., 1999).

In that respect, some data emerged from *Ricinus* studies on the effect of PCMBS on nutrient uptake and the activity of the PM H⁺-ATPase, especially during the 1980s and 1990s (Lorenc-Plucinska and Ziegler, 1987; Williams et al., 1992, 1996; Weig and Komor, 1996; Marvier et al., 1998). The effect of PCMBS on hexose transport is variable (from 0 to 70% inhibition) according to the plant material studied (Maynard and Lucas, 1982; Felker and Goodwin, 1988; Renault et al., 1992). It can also react with many PM intrinsic proteins involved in the phloem transport of acidic organic compounds, such as salicylic acid (Rocher et al., 2009) and auxin (Tamas and Davies, 2016), as well as mineral nutrient uptake, notably K⁺ (Wilkinson and Ohki, 1991; Smart et al., 1996). Therefore, it is not surprising that PCMBS reduces phloem sap exudation in *Ricinus* much more (Orlich et al., 1998) than D-GFC (Fig. 8).

**Effect of D-glucose–fenpiclonil conjugate on phloem loading of endogenous sugars in Ricinus seedlings**

Because of its high specificity, at least in the heterotrophic tissues studied (Figs 3, 4, 5 and 7), D-GFC should be a suitable tool for long-term studies (at least several hours) on endogenous sugar transport and compartmentation. Therefore, we used it to investigate the pattern of sugar phloem loading during the fifth hour after the removal of endosperm from cotyledons, i.e. when Suc in the phloem sap derives uniquely from starch breakdown (Kallarackal et al., 1989; Orlich et al., 1998). In control sets, the concentrations of fructose (which exhibited large variations due to detection limits) and glucose in the sap (Fig. 11A, B) were similar to those previously reported (Kallarackal and Komor, 1989). Glucose amounts did not change markedly in response to the different pH values of the incubation medium (from pH 5.0 to 8.0). Similarly, the Suc concentration (whose values oscillated around 160 mM from the third to the sixth hour after endosperm removal in our plant material) remained stable regardless of the pH values of the incubation medium (Fig. 11C). The conjugate at 0.5 mM did not affect total hexose concentration from pH 5.0 to pH 8.0. In contrast, it reduced the concentration of Suc in the phloem sap from 30.4% (pH 5.0) to 22.6% (pH 8.0) (Fig. 11C). The data can be analysed in relation to the pattern of solute phloem loading in *Ricinus*.

Three different pathways have been considered for Suc transport from the endosperm (or incubation solution) to the companion cell–sieve element complex: a direct apoplasmic route, a symplasmic route and an indirect apoplasmic route (Orlich and Komor, 1992; Orlich et al., 1998). This phloem loading pattern has been supported by structural, physiological, and molecular data. On the one hand, the high expression of the plasma membrane H⁺-ATPase (Williams and Gregory, 2004)
and a Suc carrier (Bick et al., 1998) in the phloem, and the relative paucity of plasmodesmata between mesophyll and bundle sheath (Orlich et al., 1998) are in agreement with a direct apoplastic component of phloem loading of Suc exported from the endosperm. On the other hand, the lower epidermis cells are modified into transfer cells (Bick et al., 1998) that possess (i) the proton pumping machinery and the Suc carrier equipment necessary for efficient sugar uptake from the endosperm (Bick et al., 1998; Williams and Gregory, 2004) and (ii) high symplasmic connections with the mesophyll allowing cell to cell transport via plasmodesmata, at least to the proximity of the bundle sheath. In addition, a transient phloem loading of Suc occurs in the presence of PCMBS (Orlich et al., 1998). These data support the involvement of a symplasmic component. Finally, time course analyses of labelled and non-labelled Suc in three compartments (mesophyll symplasm, cell-wall space and phloem exudate) indicate that an indirect apoplastic phloem loading occurs (Orlich and Komor, 1992).

Under our experimental conditions, the direct apoplastic phloem loading and the primary symplasmic route from epidermal cells cannot operate because of the removal of the endosperm for several hours (Orlich and Komor, 1992). The involvement of a supplementary symplasmic route from storage compartments is supported by the lack of effect that pH values (from 5.0 to 8.0) of the incubation medium have on the endogenous Suc concentration in the phloem sap. In addition, a contribution of the indirect apoplastic route is demonstrated by the slightly pH-dependent inhibition of endogenous Suc loading by the conjugate (Fig. 11C). Considering that the inhibition of Suc phloem loading by D-GFC is not optimal at 0.5 mM (compare Figs 3B and 9B), the contributions of the indirect apoplastic route and the symplasmic route should constitute approximately one-third and two-thirds of phloem loading, respectively, under our experimental conditions (pH 5.0, fifth hour after the removal of endosperm). Therefore our data (i) support previous work (Orlich and Komor, 1992; Orlich et al., 1998) and (ii) allow a quantitative approach to the relative contribution of these two latter ways.

Effect of D-glucose–fenpiclonil conjugate on Suc transport in two other biological models

Experiments were conducted on *Vicia faba* leaf tissues and *Saccharomyces cerevisiae* cells transformed with *AtSUC2*, which encodes the sucrose transporter involved in sucrose

### Table 2. Effect of D-GFC, PCMBS and phlorizin on the uptake of Suc and 3-O-MeG by broad bean leaf discs

| Inhibitor Substrate | Inhibition (%) | Reference |
|---------------------|---------------|-----------|
| 0.5 mM D-GFC (pH 5.0) | 0.5 mM Suc | 86.0 | Present study |
| 0.5 mM PCMBS (pH 5.0) | 0.5 mM Suc | 71.4 | Present study |
| 0.5 mM PCMBS (pH 6.0) | 1 mM Suc | 73.7 | M’Batchi and Delrot (1984) |
| 5 mM phlorizin (pH 5.0) | 1 mM Suc | 53 | Lemoine and Delrot (1987) |

In the present study, leaf discs were preincubated in a standard buffer solution (pH 5.0) for 30 min. After preincubation, the discs were transferred to the same medium solution without (control) or with 0.5 mM D-GFC or 0.5 mM PCMBS in the presence of 0.5 mM labelled Suc (specific activity: 0.20 mCi mmol⁻¹; 20 ml per 15 discs) or 3-O-MeG (specific activity: 0.30 mCi mmol⁻¹; 20 ml per 12 discs) for 30 min. Radioactivity measurements were made on each disc separately. The medians were used to calculate the inhibition percentages relative to control discs.

### Table 3. Effect of D-GFC on the uptake of Suc into transgenic Saccharomyces cerevisiae cells

Uptake of Suc into *Saccharomyces cerevisiae* cells transformed with *AtSUC2* or an empty vector in the presence of 0 mM (control), 0.25 mM, 0.5 mM, and 1 mM D-GFC for 5 min. The [¹⁴C]Suc concentration was 0.5 mM in all experiments at pH 4.5 (specific activity: 0.50 mCi mmol⁻¹).

| D-GFC concentration | Suc uptake (nmol min⁻¹ mg cells⁻¹) | Inhibition (%) |
|---------------------|-----------------------------------|---------------|
| Empty vector        |                                   |               |
| 0.19 ± 0.08         | 0.91 ± 0.03                       | 0.71          |
| 0.17 ± 0.03         | 0.55 ± 0.05                       | 0.37          |
| 0.21 ± 0.09         | 0.34 ± 0.04                       | 0.13          |
| 0.13 ± 0.02         | 0.28 ± 0.01                       | 0.15          |

The active uptake of Suc was calculated from the difference between the two *S. cerevisiae* cells (empty vector and *AtSUC2*).

The D-GFC-induced inhibition was expressed as the percentage of active uptake/control uptake.

### Table 4. Effect of D-GFC, PCMBS and phlorizin on the uptake of Suc and 3-O-MeG by broad bean leaf discs

In the present study, leaf discs were preincubated in a standard buffer solution (pH 5.0) for 30 min. After preincubation, the discs were transferred to the same medium solution without (control) or with 0.5 mM D-GFC or 0.5 mM PCMBS in the presence of 0.5 mM labelled Suc (specific activity: 0.20 mCi mmol⁻¹; 20 ml per 15 discs) or 3-O-MeG (specific activity: 0.30 mCi mmol⁻¹; 20 ml per 12 discs) for 30 min. Radioactivity measurements were made on each disc separately. The medians were used to calculate the inhibition percentages relative to control discs.

| Inhibitor Substrate | Inhibition (%) | Reference |
|---------------------|---------------|-----------|
| 0.5 mM D-GFC (pH 5.0) | 0.5 mM Suc | 86.0 | Present study |
| 0.5 mM PCMBS (pH 5.0) | 0.5 mM Suc | 71.4 | Present study |
| 0.5 mM PCMBS (pH 6.0) | 1 mM Suc | 73.7 | M’Batchi and Delrot (1984) |
| 5 mM phlorizin (pH 5.0) | 1 mM Suc | 53 | Lemoine and Delrot (1987) |
phloem loading in Arabidopsis (Stadler and Sauer, 1996). Vicia faba is typically an apoplastic phloem loader. The maturation of leaves from importing to exporting stages is characterized by a marked reduction of symplasmic connections between the phloem and the mesophyll and, within the phloem itself, by an additional symplasmic isolation of the companion cell–sieve element complex (Bourquin et al., 1990). Furthermore, in mature leaves, the companion cells are modified into transfer cells exhibiting a high expression and polarized addressing of the PM H+-ATPase (Bouché-Pillon et al., 1994). The effects of the conjugate on Suc and 3-O-MeG uptake by leaf discs were compared with data previously published concerning PCMBS (M’Batchi and Delrot, 1984) and phlorizin (Lemoine and Delrot, 1987) (Table 2). The dramatic inhibition of Suc uptake induced by 0.5 mM D-GFC in Ricinus was observed again using Fabaceae leaf discs. By comparison, 5 mM phlorizin is a relatively poor inhibitor. The effect of D-GFC in Fabaceae was not as specific as in Ricinus because of its slight effect on 3-O-MeG uptake. Nevertheless, considering the Suc/3-O-MeG inhibition ratio, its specificity is better than that of PCMBS and clearly of phlorizin (Table 2).

Transgenic Saccharomyces cerevisiae cells transformed with AtSUC2 constituted an elegant model to study the effect of D-GFC on Suc uptake by the H+-Suc symporter involved in Suc loading in Arabidopsis (Stadler and Sauer, 1996; Gottwald et al., 2000). At a concentration as low as 0.25 mM, the active component of the Suc uptake was inhibited by approximately 50% in 5 min. The maximum inhibition plateaued (approximately 80%) at 0.5 mM (Table 3).

These data indicate that the potent inhibitory effect of D-GFC on sucrose carriers is not limited to cotyledons of seedlings with endosperm, i.e. heterotrophic tissues that function as the small intestinal wall (Robinson and Beevers, 1981a). The effect of D-GFC is similar to that of PCMBS in apoplastic loaders and much higher than the natural glucose side phlorizin (Lemoine and Delrot, 1987; Bush, 1993).

Conclusion
Using different biological models (heterotrophic cotyledon tissues, mature exporting leaves, and transgenic Saccharomyces cerevisiae cells) from different plant families (Euphorbiaceae, Fabaceae, and AtSUC2 from Brassicaceae), our data show that D-GFC is an inhibitor of Suc carriers as potent as PCMBS in acidic conditions. This xenobiotic glucoside reversibly blocks the H+-Suc symporters involved in Suc exchanges at strategic sites of the plant, namely at the triploid endosperm–cotyledon tissues interface and phloem loading in cotyledons, as well as phloem loading in mature leaves of apoplastic loaders. While PCMBS forms covalent bonds with sulphydryl groups of many PM intrinsic proteins and therefore affects uptake and phloem transport of many solutes in addition to Suc, D-GFC can exhibit much more selectivity, especially in Ricinus seedlings and possibly in other seedlings with endosperm. Unlike PCMBS, this new tool in phloem biology allows long-term phloem exudation and therefore investigation, with quantitative analysis, of the pathways involved in phloem loading of endogenous Suc in Ricinus, as evidenced in the present work. The use of D-GFC can be extended to the study of sugar exchange between vascular tissue apoplasm and symplasm in response to abiotic stresses.

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