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Carrier-mediated transport of salicylic acid

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Salicylic acid transport in *Ricinus* involves a pH dependent carrier system in addition to diffusion

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Abstract

Despite its important functions in plant physiology and defence, membrane transport mechanism of salicylic acid (SA) is poorly documented due to the general assumption that SA is taken up by plant cell via the ion trap mechanism. Using *Ricinus* seedlings and modelling tools (ACD LogD and Vega ZZ softwares), we show that phloem accumulation of SA and hydroxylated analogs is completely uncorrelated with the physicochemical parameters suitable for diffusion (number of hydrogen bond donors, polar surface area and especially, LogD values at apoplastic pHs and Δ LogD between apoplast and phloem sap pH values). These and other data (such as accumulation in phloem sap of the poorly permeant dissociated form of monohalogen derivatives from apoplast, inhibition of SA transport by the thiol reagent pCMBS) lead to the following conclusions. As in intestinal cells, SA transport in *Ricinus* involves a pH dependent carrier system sensitive to pCMBS; this carrier can translocate monohalogen analogs in the anionic form; efficiency of phloem transport of hydroxylated benzoic acid derivatives is tightly dependent on the position of the hydroxyl group on the aromatic ring (SA corresponds to the optimal position) but moderately affected by halogen addition in position 5 which is known to increase plant defence. Furthermore, combining time course experiments and pCMBS used as a tool, we give information about the localisation of the SA carrier. SA uptake by epidermal cells (i.e. the step preceding the symplastic transport to veins), insensitive to pCMBS, occurs via the ion trap mechanism whereas apoplastic vein loading involves a carrier mediated mechanism (which is targeted by pCMBS) in addition to diffusion.

Salicylic acid (SA), classified under the group of plant hormones, has been reported to induce heat production in inflorescences of thermogenic species, stimulate flowering and enhance flower longevity, inhibit ethylene biosynthesis, block the wound response and reverse the effect of abscissic acid (Davies, 2004; Hayat et al., 2007). Moreover, considerable attention has been focused for about two decades on the role of SA in plant disease resistance since the observation that acetylsalicylic acid (aspirin) induces resistance to viruses and is highly effective in activating pathogenesis related genes (White, 1979). Further supports for the role of SA in plant disease resistance come from mutants and transgenic plants impaired in the accumulation of SA and thereby unable to develop efficient defence responses after infection (Gaffney et al., 1993; Ryals et al., 1996; Sticher et al., 1997) and measurements of endogenous SA levels in response to pathogen attacks (Malamy et al., 1990; Yalpani et al., 1991). Although free SA is not the primary signal that induces systemic acquired resistance (SAR), (Rasmussen et al., 1991; Vernooij et al., 1994; Ryals et al., 1996; Maldonado et al., 2002; Park et al., 2007; Truman et al., 2007) it also moves from the infected leaf to the upper uninoculated leaves via the sieve tubes (Metraux et al., 1990; Yalpani et al., 1991; Shulaev et al., 1995; Molders et al., 1996). It may be supposed that, in mature infected tissues, SA transport to the sieve elements occurs via either the symplastic or the apoplastic pathway according to the species, as described in the past for assimilates (Bonnemain, 1975). In the case of apoplastic phloem loading, SA must cross the plasma membrane of the companion cell-sieve element complex. Similarly, exogenous SA and analogs applied to the leaf surface must cross the plasma membrane of a cell to reach the phloem sap. Because SA is a weak acid, it is generally thought that SA phloem uptake occurs by diffusion of its lipophilic undissociated form which dissociates into poorly permeating anions into the phloem symplast (Krasavina, 2007). According to Kleier's predicting model, the physical properties of this hormone, in terms of pKa values and octanol/water partitioning coefficient ($\log K_{ow}$), are nearly ideal for phloem systemicity by way of the ion-trap mechanism (Yalpani et al., 1991).

SA, under its pharmaceutical derivative (aspirin) is a popular drug. It was initially believed that monocarboxylic acid drugs were absorbed from the small intestine lumen by a passive diffusion mechanism depending on the degree of protonation of the carboxylate moiety and the lipid solubility of the unionized molecule (Brodie and Hogben, 1957) in the "acid microclimate" (pH 5.5-6.0) on the intestinal surface (Elbert et al., 1986). Then experiments conducted with brush-border membrane vesicles from animal small intestine or the human colon adenocarcinoma cell line, Caco-2 cells, which exhibits several functional properties of small intestine, have demonstrated that transport of various monocarboxylic

acids (acetic acid, nicotinic acid, benzoic acid, salicylic acid) occurs mainly via a pH dependent and carrier-mediated transport mechanism (Simanjuntak et al., 1990; Takanaga et al., 1994; Tsuji et al., 1994). These works led to the characterization of monocarboxylate transporter (MCT) family in animal cells (Garcia et al., 1994; Tamai et al., 1995; Enerson and
5 Drewes, 2003).

In a recent paper (Rocher et al., 2006), we have studied the ability of the phloem of *Ricinus* seedlings to load exogenous SA. It was shown that phloem loading of SA is dependent on the pH of the cotyledon incubating medium and is the highest at the most acidic values (pH 4.6 and 5.0). However, a residual phloem loading still occurs at pH values close to
10 neutrality, i.e. when SA is only in its poorly permeant dissociated form. Furthermore, in contrast with the Kleier and Bromilow model predictions (Bromilow et al., 1989, 1991; Hsu and Kleier, 1996; Kleier and Hsu, 1996), dichlorinated analogs (with a Log Kow near 4) also moved in the phloem. These discrepancies may give rise to the hypothesis that SA and aromatic monocarboxylate analogs are taken up by a carrier system in addition to the ion-trap
15 mechanism (Rocher et al., 2006). These data need further investigations. Particularly, Log Kow values refer to molecules in their neutral form which may not exist or be present only in small part (as SA) at biological pH. In this regard, at this step of the study, important parameters controlling diffusion and ion trap mechanism (number of hydrogen bond donors, polar surface area, Δ LogD between apoplast and phloem sap pH values) were not taken into
20 consideration and there was a lack of direct physiological evidence that SA transport is carrier mediated. The purpose of the present work is to test this hypothesis. Here, we demonstrate that phloem transport of SA and its halogen analogs is carrier mediated, put forward several functional properties of the carrier system, especially its substrate specificity and its sensitivity to the thiol reagent pCMBS. We also give information about the tissular
25 localisation of this transporter and give details of the mechanisms of the two pathways involved in SA phloem loading.

RESULTS AND DISCUSSION

Predicting phloem mobility of SA analogs using Kleier and Bromilow and co-workers models

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The phloem mobility of three types of SA analogs was studied (Table I), firstly monohalogenated derivatives with an halogen addition in position 5; secondly benzoic acid (BA) and BA derivatives differing from SA by the position of the hydroxyl group; and

thirdly, an endogenous compound with the carboxylic function masked by a methyl group, i.e. methyl salicylate (MeSA).

As previously (Rocher et al., 2006), phloem mobility was predicted using the models of Kleier (Kleier, 1988; Hsu and Kleier, 1996) and Bromilow and co-workers (Bromilow et al., 1991). These models indicate two physicochemical properties (pKa and Log K_{ow} values) of xenobiotics and natural compounds required for phloem accumulation (Hsu and Kleier, 1996) or various types of systemic behaviour (Bromilow et al., 1991)(Fig. 1). Because several parameters, such as penetration across leaf cuticle, metabolism and cell compartmentation are not taken into consideration, the aim of these models is to give useful information of systemicity ability of compounds rather than accurate predictions of mobility (Bromilow and Chamberlain, 1995; Hsu and Kleier, 1996). In this regard, *Ricinus* seedlings with their thin and highly permeable cuticle which facilitates molecule diffusion to and therefore within the cotyledon apoplast is the suitable plant material to test the information from the predicting models. These latter have been also used to design phloem mobile propesticides (Kleier and Hsu, 1996; Kleier, 2001; Chollet et al., 2005). Furthermore, strong discrepancies between predictions and experimental data may indicate the involvement of a carrier system instead of diffusion through the plasma membrane (Oparka, 1991).

According to the calculations of pKa and Log K_{ow} made by ACD LogD suite version 11.01 software, all the SA analogs exhibited a phloem mobility ability near to that of SA in both models (Fig. 1), except MeSA which was predicted to be non mobile (Fig. 1A) or mobile only in the xylem (Fig. 1B).

Predicting transport of SA and SA analogs using diffusion predictors

Two parameters are now intensively used for the prediction of diffusion of small molecules (MW < 500 Da) through the human membranes : the polar surface area (PSA) and the number of hydrogen bond donors (HBD) (Winiwarter et al., 2003; Ertl, 2008). HBD has been also assayed in algae (Raevsky and Schaper, 1998). PSA is defined as the sum of surfaces of polar atoms in a molecule, usually oxygen and nitrogen and the hydrogens bonded to these atoms (Ertl et al., 2000). This descriptor has been found to correlate well with passive drug permeability through the plasma membrane and, therefore, allows reliable predictions of transport properties of drug candidates in clinical development (Palm et al., 1998; Winiwarter et al., 1998) except for actively transported molecules (Palm et al., 1997; Winiwarter et al., 1998). To diffuse easily through the intestinal barrier, molecules must exhibit a PSA less than 60 Å².

The second descriptor for the prediction of absorption of drugs is the hydrogen-bonding capacity and particularly the number of hydrogen bond donors (HBD), ie a hydrogen atom attached to a relatively electronegative atom. HBD was successfully used for the correlation with permeability and absorption data for numerous chemicals and drugs in human cells (Raevsky and Schaper, 1998; Winiwarter et al., 1998; Winiwarter et al., 2003) and algae ((Raevsky and Schaper, 1998). Passive absorption is more likely when there are less than 5 (Lipinski et al., 1997) or 10 (Palm et al., 1997) HBD per molecule.

SA and SA analogs have low PSA and HBD values, consistent with efficient diffusion (Table I). Moreover, these values are identical (HBD) or very close (PSA) for SA, 3-OHBA and 4-OHBA. If the passive transport was the only mechanism involved in *Ricinus* phloem uptake, the concentration factor should be in the same range for all three positional isomers.

PSA and HBD are considered as more convenient diffusion predictors than LogD from intestinal lumen (Winiwarter et al., 2003). There is an interesting analogy about the pH gradients values between the surface of intestinal cells (pH 5.5-6.0) and the blood (pH 7.35-7.45) and the pH gradients between the phloem apoplast and the phloem sap. In absence of buffers, the tissues of the *Ricinus* cotyledons stabilize the incubation medium at pH 5.2 (our measurements) while the pH values of the *Ricinus* phloem sap vary from 7.5 to 8.2 (Hall and Baker, 1972; Vreugdenhil and Koot-Gronsveld, 1989). Taking into account these latter high pH gradients, LogD of ionisable compounds and Δ LogD variations from apoplast to phloem sap pH values (Δ LogD_{A-P}) need a special attention.

Structure - phloem mobility relationships

Halogen addition in position 5 on the aromatic ring which enhances elicitor response against pathogens (Kauss et al., 1993) slightly (5-ClSA) or marginally (5-FSA) increases molecular weight and the molar volume. These molecules are less hydrophilic and less dissociated than SA at acidic pH values but LogD variations from apoplast to phloem sap pH values are very small (Δ LogD_{A-P} \approx 0.25) (Table I, Fig. 2A and 2B). They exhibited good phloem mobility, although less than that of SA (concentration factor near 5 at pH 4.6 and 5.0 instead of 9 for SA) (Fig. 3A and B). Phloem mobility of both compounds was pH dependent, i.e. influenced by pH of the solution (Fig. 3A and B) but not correlated to the percentage of the permeant undissociated form at the octanol-water interface (Fig. 2B) as calculated with ACD LogD software. A residual phloem transport (5-ClSA) or a clear phloem accumulation (5-FSA; concentration factor = 2) was noted at external pH 7.0 and 8.2 when these molecules were fully dissociated and at there minimum permeability for partitioning into the bilayer.

This accumulation of 5-FSA in its anionic form in the phloem sap is completely inconsistent with diffusion. A residual uptake of BA and SA at pH values close to neutrality was also noted in intestinal cells (Takanaga et al., 1994; Tsuji et al., 1994). By contrast, phloem uptake of acid derivatives of fenpiclonil in *Ricinus* occurred only in their permeant undissociated form (Chollet et al., 2004, 2005).

BA, 3-OHBA and 4-OHBA exhibited a much better physicochemical profile than SA for diffusion through a phospholipidic layer in response to a transmembrane pH gradient in terms of LogD at acidic pHs, LogD variations from apoplast to phloem sap pH values ($\Delta \text{LogD}_{\text{A-P}} \approx 2.5$ instead of 0.5 for SA) and percentage of the permeant undissociated form at acidic pHs (Table I, Fig. 2C and D). It may be added that in a pure water medium, the percentage of the permeant undissociated form was 29, 19 and 41 % at pH 4.6 for BA, 3-OHBA and 4-OHBA respectively while SA monohalogenated analogs were only in their dissociated form according to the calculations made with ACD LogD sol suite software. However, the phloem loading of these compounds, especially that of 3-OHBA (concentration factor = 0.70 at pH 4.6) and 4-OHBA (concentration factor = 0.95 at pH 4.6), was dramatically reduced in comparison to that of SA, 5-ClSA and 5-FSA (Fig. 3 and 4) and this cannot be explained by PSA and HBD values (Table I) which are the same for the three molecules as mentioned above. These strong discrepancies, and those mentioned above, between predictions of diffusion through the plasma membrane (Table I, Fig. 1 and especially Fig. 2) and the experimental data (Figs. 3 and 4) strongly indicate that SA phloem loading involves a pH-dependent carrier system in addition to the ion-trap mechanism.

MeSA can diffuse through the membrane taking into account its PSA and HBD values (Table I) but similarly in both directions because the molecule is not ionisable at biological pH values ($\Delta \text{LogD}_{\text{A-P}} = 0$). In agreement with the predicting models of phloem mobility (Fig. 1A and B) MeSA concentration in the phloem sap was so low that it could not be detected by HPLC in the *Ricinus* phloem sap (under our experimental conditions, the detection limit for MeSA was 0.25 μM concentration). This is consistent with recent measurements of MeSA levels by GC-MS in tobacco leaves. MeSA concentrations vary from about 3 nM to 10 nM in the phloem sap according to the plant material and treatments and are about 100-fold lower than those noted in the leaf tissue (Park et al., 2007). By contrast, after leaf inoculation with the tobacco necrosis virus, SA concentrations vary from about 0.5 to 7 μM in the mesophyll and from 2 to 9 μM in the phloem sap (Malamy et al., 1990; Mettraux et al., 1990) despite SA metabolism and SA 2-O- β -D-glucoside sequestration in the vacuoles. SA levels can even

reach 200 to 500 μM in the sap after *Pseudomonas* infection of cucumber leaves (Rasmussen et al., 1991).

Our data show that efficient phloem transport of BA derivatives requires a free carboxyl group on the aromatic ring and a hydroxyl group in position 2. It has been recently demonstrated that some sucrose carriers (AtSUC9, LjSUT4) transport several phenyl glucosides including salicin (2-(hydroxymethyl)phenyl- β -D-glucopyranoside) (Sivitz et al., 2007; Reinders et al., 2008) which is metabolized to SA in the human body, but not SA (Sivitz et al., 2007). Furthermore, complementary experiments indicated that SA was not translocated by ANT1, an aromatic and neutral amino acid carrier also transporting non hydroxylated monocarboxylic acid such as auxin and 2,4-D (Chen et al., 2001) (Fig. 5). This is in agreement with the conclusion that specific structural requirements must be met for efficient transport of aromatic monocarboxylic acids (Fig. 4). By contrast, the animal monocarboxylate transporter MCT1 which translocates SA is also implicated in intestinal absorption of several endogenous and exogenous compounds differing in size and structure, from unbranched aliphatic monocarboxylates such as acetate and propionate to much larger molecules such as statins (Enerson and Drewes, 2003; Halestrap and Meredith, 2004).

Effect of p-chloromercuribenzenesulphonic acid (pCMBS) on SA uptake by *Ricinus* cotyledons

This thiol reagent have been widely used to study nutrient uptake for a long time (Giaquinta, 1977). Due to its physicochemical properties, it is considered as a poorly or non-permeant tool and this is consistent with experimental data indicating that pCMBS is membrane impermeable (Bourquin et al., 1990; Bush, 1993). For instance, infiltration of broad bean leaf apoplast by 2 mM pCMBS completely blocks phloem loading of assimilates without affecting significantly CO_2 assimilation nor the transmembrane potential difference generated by the plasma membrane H^+ -ATPase (Bourquin et al., 1990). pCMBS is known as a potent inhibitor of sucrose and oligopeptide carriers by reacting with Cys residues in the external part of these transporters (Delrot et al., 1980; Bush, 1993; Jamai et al., 1994; Orlich et al., 1998; Lemoine, 2000; Knop et al., 2004). This inhibiting effect cannot be generalized to other nutrient carriers, such as monosaccharide transporters (Noiraud et al., 2001) and depends on the location of Cys residues in the extracellular domains (Ramsperger-Gleixner et al., 2004).

At acidic pHs, SA uptake by *Ricinus* cotyledons was significantly inhibited by 1 mM pCMBS (Fig. 6). By contrast, diffusion of the weak acid ^{14}C -DMO, used as internal pH probe,

through the plasma membrane in response to the transmembrane pH gradient was exactly the same in control and treated sets (Fig. 6). This indicates that the ion trap mechanism of weak acids is not affected in presence of pCMBS and that a protein-mediated translocation pathway for SA is targeted by the thiol reagent. The localization of ^{14}C -SA (and labelled metabolites) in cotyledons tissues was very similar to that of ^{14}C -sucrose. It has been previously shown that ^{14}C -sucrose was taken up not only by the phloem but also by the epidermal cells of *Ricinus* seedlings (Martin and Komor, 1980; Weig and Komor, 1996). Consequently, the labelling of cotyledons epidermis and mesophyll masked the minor veins on autoradiographic pictures (Martin and Komor, 1980). The distribution of ^{14}C -SA exhibited a similar pattern (compare Fig. 7A and D) and the minor vein network was hardly perceptible. pCMBS affected the general labelling of the tissues, but particularly the vein network. Most of the major veins were no more perceptible (Fig. 7B and C). Sucrose phloem loading was also inhibited by pCMBS as well as sucrose uptake by external tissues (Fig. 7D and E). These data indicate that SA transport in *Ricinus* tissues involves a carrier system sensitive to pCMBS. Within the animal monocarboxylate transporter family, some carriers such as MCT1 are sensitive to pCMBS while others are not (Halestrap and Meredith, 2004).

Localisation of the SA carrier targeted by pCMBS : mechanisms of the two routes of SA phloem loading

The castor bean is a symplastic-apoplastic loader (Orlich and Komor, 1992). This means that endogenous molecules from seedling endosperm (or exogenous compounds from an incubation solution) found in the phloem sap may come, via the symplastic pathway, from the transfer cells of the lower epidermis, or may be taken up directly from the phloem apoplast. This is the case with sucrose which is translocated by the sucrose transporter Rcscr1 and RcSUT1, the latter being located both in lower epidermis and in the phloem (Weig and Komor, 1996; Bick et al., 1998). In this regard, time course of ^{14}C -sucrose uptake into *Ricinus* cotyledons, as measured by autoradiography, suggest that the epidermis primarily take up sucrose. The labelling of the major veins can be detected only after a 5 min incubation period and became obvious after 10 min (Martin and Komor, 1980). On this basis, the effect of pCMBS on ^{14}C -SA uptake by cotyledons tissues was studied after a 3 min incubation period, ie when the labelled molecules diffused mainly within the apoplast of the external cell layers, and after a 10 min incubation period.

Under the shortest experimental conditions, ^{14}C -SA uptake was similar in the control and in the presence of pCMBS while ^{14}C -sucrose uptake was dramatically inhibited by the

thiol reagent (Fig. 8). This means that the SA carrier targeted by pCMBS is not located in the outer cells of *Ricinus* cotyledons (and especially in the lower epidermis transfer cells) contrarily to the sucrose carrier system. By contrast, after a 10 min incubation, both SA and sucrose uptake were inhibited (about 50 % and 90 % respectively at pH 5.0) by the thiol reagent (Fig. 9). Moreover, autoradiograph pictures showed that the major vein labelling was practically abolished in the treated set (Fig. 10). These data indicate that the SA carrier system targeted by pCMBS is mainly located in the vein area. A complementary experiment was conducted at pH 7.0 to examine the pH dependence of the pCMBS inhibiting effect on SA uptake. The apparent inhibition varied from 27 % (pH 4.6) to 74 % (pH 7.0) (Fig. 10). This suggests that the true inhibition of the SA carrier system by the thiol reagent at the acidic pH values, especially at pH 4.6, is masked by the contribution of the ion trap mechanism to the over-all uptake. At pH 5.0, i.e. a pH value close to that of cotyledons epidermis apoplast, the contribution of the ion trap mechanism to the over-all uptake seems not very different to that of the carrier mediated mechanism (Fig. 9). The strong inhibition of SA uptake at pH 7.0 also supports that the carrier can manipulate SA in its anionic form.

Finally, our data indicate that SA molecules moving from epidermal transfer cells to the phloem via the symplastic route are taken up by a mechanism insensitive to pCMBS (Fig. 8), logically the ion trap mechanism. The molecules which are not metabolized and sequestered in the vacuole during their transport from cell to cell can reach the phloem. SA molecules which are directly taken up from the apoplast in the vein areas are translocated by a carrier system sensitive to pCMBS (Fig. 9) and accumulate in the phloem. The ion trap mechanism contribution to a direct phloem loading of the conducting cells from the phloem apoplast appears to be a minor component of the over-all phloem loading, taking into account the dramatic inhibition of conducting tissues labelling noted in presence of pCMBS (Fig. 10).

CONCLUSION

Combining i/ the predictions of phloem mobility of SA and SA analogs from the Kleier *et al.* and Bromilow *et al.* models, ii/ software calculations of physicochemical parameters of these molecules, such as PSA, HBD and especially LogD and percentage of the undissociated form and their variations according to the pH values of apoplastic compartment and phloem sap, iii/ analyses of phloem sap from a simple plant model without cuticle barrier, which give access to phloem plasma membrane properties and iv/ the use of a thiol reagent, it has been possible to demonstrate that SA transport involves a pH dependent carrier system that is

sensitive to pCMBS. This is the first time that occurrence of a carrier system in plant tissues is predicted by analysis of the discrepancies between the predictions of computational models and the actual results of the experiments. Furthermore, the rather poor phloem mobility of 3-OHBA and 4-OHBA indicates that this carrier system exhibits high substrate specificity in regard to the relative position of the carboxyl and hydroxyl groups.

Then combining time course experiments, pCMBS used as a tool and autoradiograph inhibition studies, it has been possible to pinpoint the tissular localization of the SA carrier in *Ricinus* cotyledons. Contrarily to the sucrose carrier system which is expressed both in the epidermis and the veins (Bick et al., 1998), the SA carrier targeted by pCMBS is located only in the inner tissues, especially in the veins. As *Ricinus* is an apoplastic-symplastic loader (Orlich and Komor, 1992), SA phloem loading involves three components: i/ a symplastic transport from the outer cells especially the epidermis transfer cells, ii/ an uptake from the phloem apoplast via the ion trap mechanism and, iii/ an uptake from apoplast mediated by a carrier system which is targeted by pCMBS.

MATERIAL AND METHODS

Plant material

Castor bean seeds (*Ricinus communis* L. cv Sanguineus), obtained from Ball-Ducrètet (Margencel, France) were placed in wet cotton wool for 24 h at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$ prior to sowing in vermiculite watered with tap water. Seedlings were grown in a humid atmosphere ($80\% \pm 5\%$) at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Phloem sap collection and analysis

The sap collection method was similar to that recently described (Rocher et al., 2006). The phloem sap was analyzed by HPLC after dilution with UHQ grade water (1 + 9 v/v). We employed reversed phase chromatography using a Discovery RP-amide C16 column (length 250 mm, internal diameter 4.6 mm) (Supelco, Bellefonte, PA) or a Chromolith performance RP 18e column (length 100 mm, internal diameter 4.6 mm) (Merck, Darmstadt, Germany) in accordance with the procedure set out in Table II. Results were processed with PC 1000 software v3.5 from Thermo Fisher Scientific (Courtabœuf, France).

Uptake in cotyledons discs

Discs (1.13 cm² surface) were obtained with a 12 mm diameter cork borer from *Ricinus* cotyledons. Then, they were floated on a preincubation medium containing 20mM 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid [HEPES] (pH 4.6, 5.0, 7.0) as buffer, 0.5mM CaCl₂ and 0.25 mM MgCl₂. After a 30min preincubation period, the discs were incubated in the same buffered solution containing either 10 μM [¹⁴C]-SA or 10 μM [2-¹⁴C]-DMO or 100 μM [¹⁴C]-Suc with or without pCMBS at 1 mM concentration. Incubation was run under mild agitation on a reciprocal shaker at room temperature. After 3, 10 or 60 min incubation, the disc apoplast was rinsed (3 x 2 min) in a solution similar to the preincubation medium. Then, each disc was digested overnight at 55°C in a mixture of perchloric acid (65%; 25μl), hydrogen peroxide (33%; 50μl) and Triton X-100 (1g.l⁻¹; 50μl). After adding 4 ml of scintillation liquid (Ecolite + from MP Biomedicals), the radioactivity was counted by liquid scintillation spectrometry (Packard Tricarb 1900TR). In other sets, discs were incubated with 10 μM [¹⁴C]-SA or 100 μM [¹⁴C]-Suc with or without pCMBS. After 10 or 60 min incubation, the discs were rinsed, dry-ice frozen, lyophilized and autoradiographed (Kodak Biomax MR film).

Uptake in *Saccharomyces* expressing ANT1

Salicylic acid transport by an aromatic and neutral amino acid transporter from Arabidopsis was tested. For this purpose, the ANT1 transporter expressed in yeast strain JT16 was used (Chen et al., 2001). Experiments were conducted as described in (Noiraud et al., 2001) with ¹⁴C-salicylic acid at an external concentration of 50 μM. ³H-valine at a 100 μM concentration was used as a control. Results were the difference between uptake of yeasts expressing ANT1 and yeasts transformed with the empty plasmid. Each experimental point was repeated 4 times.

Chemicals

The compounds to be added to incubation solutions were from Acros Organics (Noisy-le-Grand, France) (salicylic acid, benzoic acid, 5-chlorosalicylic acid, 5-fluorosalicylic acid, 4-morpholinoethanesulfonic acid [MES], 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid [HEPES], from Alfa-Aesar (Karlsruhe, Germany) (3-hydroxybenzoic acid, 4-hydroxybenzoic acid), from Sigma-Aldrich Chimie (St Quentin Fallavier, France) (salicylic acid-carboxy-¹⁴C), from American Radiolabeled Chemicals (St Louis, USA) (5,5'-dimethyl-

oxazolidine-2-¹⁴C,4-dione [DMO]) and from Toronto Research Chemicals (North York, Canada) (4-(chloromercuri)benzenesulfonic acid, sodium salt [pCMBS]).

Physicochemical properties

5 Physicochemical properties and descriptors of salicylic acid and other ionizable molecules were predicted using ACD LogD Sol Suite v 11.01 software from Advanced Chemistry Development, Inc. (Toronto, Canada). This package of programs calculates Log K_{ow} (ie the pH-independent octanol-water partition coefficient), pKa (ionization constant in aqueous solution), solubility and dissociation in water at any pH, LogD (i.e. the pH dependent
10 Log K_{ow}) and the number of hydrogen bond donors (HBD). To calculate LogD (ie the partition coefficient for almost any drawn organic compound at any pH), the software uses both pKa and Log K_{ow} information as already mentioned (Rocher et al., 2006). The algorithms for the predictions are based on contributions of separate atoms, structural fragments and intramolecular interactions between different fragments. LogD is a very important parameter
15 considered for bioavailability and absorption studies of drugs (van de Waterbeemd et al., 2003) and agrochemicals (Chollet et al., 2004, 2005). The calculation of confidence limits for LogD (δ LogD) is not implemented into ACD software but can be evaluated for a mono-acid with the following formula:

$$\delta \log D = \delta \log P_{ion} \cdot \left| \frac{10^{\log P_{ion}}}{10^{\log P_{ion}} + 10^{(pKa-pH+\log P)}} \right| + \delta pKa \cdot \left| \frac{10^{(pKa-pH+\log P)}}{10^{\log P_{ion}} + 10^{(pKa-pH+\log P)}} - \frac{10^{(pKa-pH)}}{1 + 10^{(pKa-pH)}} \right| + \\ + \delta \log P \cdot \left| \frac{10^{(pKa-pH+\log P)}}{10^{\log P_{ion}} + 10^{(pKa-pH+\log P)}} \right|$$

20 where $\log P$ is the octanol-water partition coefficient for uncharged species and $\log P_{ion}$ is the octanol-water partition coefficient for negatively charged species.

Molecular volume and polar surface area (PSA) were computed after running Mopac semi-empirical calculations with PM3 parameters using Vega ZZ v. 2.3.1 software from Drug Design Laboratory (Milano, Italy).

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FIGURE LEGENDS

Figure 1. Prediction of phloem mobility of SA, 5-ClSA, 5-FSA, BA, 3-OHBA, 4-OHBA and MeSA. (A), Kleier map (Log Cf as a function of Log Kow and pKa) according to Kleier et al (1996); plant parameters are for a short plant. (B), Bromilow model (degrees of mobility as a function of Log Kow and pKa) according to Bromilow et al (1991).

Figure 2. Physicochemical properties of SA and SA derivatives. LogD of SA, 5-ClSA, 5-FSA (A) and SA, BA, 3-OHBA, 4-OHBA (C) and percentage of undissociated form of SA, 5-ClSA, 5-FSA (B) and SA, BA, 3-OHBA, 4-OHBA (D) in an octanol/water mixture as a function of pH. The apoplast and phloem sap pH values are dash grey and light grey respectively. Note that Δ LogD variations between pH 5.0 and 8.0 are small for SA and halogen derivatives (about 0.5 and 0.25 unit respectively) and high for BA, 3-OHBA, 4-OHBA (about 2.5 units). The apoplastic pH values are from (Giaquinta, 1977) but more acidic values are possible in phloem apoplast taking into consideration the high expression of the PM H⁺-ATPase in phloem cells, especially in companion cells (Bouche-Pillon et al., 1994). SA LogD values and percentage of undissociated form calculated by ACD LogD v 11.01 are similar to those calculated by version 9.0 (Rocher et al., 2006).

Figure 3. Phloem mobility of SA halogen derivatives. Concentration factor of 5-ClSA (A) and 5-FSA (B) (added to the incubation solution at 10 μ M, final concentration) in phloem sap of *Ricinus* as a function of the pH of the incubation medium. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [5-ClSA or 5-FSA]_{sap} / [5-ClSA or 5-FSA]_{medium}. Box plots, $10 \leq n \leq 17$.

Figure 4. Phloem mobility of SA and SA analogs. Concentration factor of SA, BA, 3-OHBA, 4-OHBA and MeSA (added to the incubation medium at 10 μ M, final concentration) in phloem sap of *Ricinus*. Cotyledons were incubated in a buffered solution at pH 4.6 or 6.0. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [studied product]_{sap} / [studied product]_{incubation medium}. MeSA could not be detected. Box plots, $16 < n < 29$.

Figure 5. Time dependent uptake of 100 μ M [³H]-Valine and 50 μ M [¹⁴C]-SA by yeast strain JT-16 expressing the amino acid transporter ANT1 from *Arabidopsis thaliana* (Chen et al., 2001). Data are the mean of 4 replicates and are expressed as the difference between uptake in JT-16/ANT1 and JT-16/pYES2 (empty plasmid) to measure transport due to ANT1.

Figure 6. Effect of 1 mM pCMBS on 10 μ M SA or 10 μ M DMO uptake by discs from *Ricinus* cotyledons. Discs were pre-incubated in a buffered solution (pH 4.6 or pH 5.0) for 30

min and then transferred to the incubation medium for 1 h (same solution containing [^{14}C]-SA or [^{14}C]-DMO without [grey boxes] or with [white boxes] pCMBS). The Mann-Whitney U test was used to assess statistically significant differences (** $p < 0.001$; NS not significant). Boxplots, $10 < n < 16$.

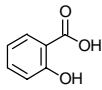
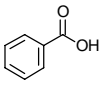
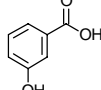
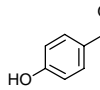
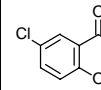
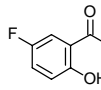
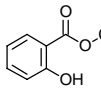
- 5 **Figure 7.** Autoradiographic pictures of the lower side of *Ricinus* cotyledons. Discs were incubated for 1 h in a buffered solution (pH 5.0) containing either 10 μM [^{14}C]-SA (A and B; exposure time 15 h) or 100 μM [^{14}C]-sucrose (D and E; exposure time 51 h) without (A and D) or with (B and E) 1 mM pCMBS. C was a photography of the major veins of the cotyledon area autoradiographed in B. Radioactivity appears in black.
- 10 **Figure 8.** Effect of 1 mM pCMBS on 10 μM SA or 100 μM sucrose uptake by discs from *Ricinus* cotyledons for 3 min. Discs were floated on a buffered standard solution (pH 5.0) for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (same solution containing either 10 μM [^{14}C]-SA or 100 μM [^{14}C]-sucrose without [grey boxes] or with [white boxes] pCMBS) for 3 min. The Mann-Whitney U test was used to assess statistically significant differences (** $p < 0.001$; NS not significant). Boxplots, $n = 10$.
- 15

- Figure 9.** Effect of 1 mM pCMBS on 10 μM SA uptake or 100 μM sucrose uptake (insert) by discs from *Ricinus* cotyledons for 10 min. Discs were pre-incubated in a buffered standard solution for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min.
- 20 After the pretreatment, tissues were transferred to the incubation medium (same solution containing 10 μM [^{14}C]-SA or 100 μM [^{14}C]-sucrose without [grey boxes] or with [white boxes] pCMBS) for 10 min. The Mann-Whitney U test was used to assess statistically significant differences (** $p < 0.001$; ** $p < 0.01$). Boxplots, $12 < n < 16$.

- Figure 10.** Autoradiographic pictures of the lower side of *Ricinus* cotyledons. Discs were floated for 30 min on a standard preincubation medium and then on the same medium for an additional 15 min without (A) or with (B) 1 mM pCMBS. After the pretreatment, tissues were incubated for 10 min in the same buffered solution (pH 5.0) containing 10 μM [^{14}C]-SA without (A) or with (B) 1 mM pCMBS. Exposure time was 100 h. Radioactivity appears in black. Major veins are underlined by arrowheads.
- 25

Table I. Chemical structure and physicochemical descriptors (molecular weight, molecular volume, pKa, log D ie the pH-dependent partition coefficient, percentage of protonated form, polar surface area and number of hydrogen bond donors) of SA and other derivatives used in this work. All parameters were computed using ACD LogD suite v 11.01 software except

5 molecular volume and polar surface area which were estimated using Vega ZZ v 2.3.1.

Compound Name		Salicylic acid (SA)	Benzoic acid (BA)	3-Hydroxybenzoic acid (3-OHBA)	4-Hydroxybenzoic acid (4-OHBA)	5-Chlorosalicylic acid (5-CISA)	5-Fluorosalicylic acid (5-FSA)	Methyl salicylate (MeSA)
Structural Formula								
Molecular Weight (Da)		138.12	122.12	138.12	138.12	172.57	156.11	152.15
Molecular Volume (Å ³)		115.9	108.6	116.1	115.7	130.0	121.0	132.6
pKa ₁ (COOH)		3.01 ± 0.1	4.20 ± 0.1	4.08 ± 0.1	4.57 ± 0.1	2.64 ± 0.1	2.68 ± 0.1	-
log D	pH 4.6	-0.34 ± 0.36	1.02 ± 0.28	0.5 ± 0.30	1.01 ± 0.27	0.54 ± 0.39	0.045 ± 0.47	2.52 ± 0.24
	pH 6.0	-1.06 ± 0.51	-0.23 ± 0.33	-0.78 ± 0.34	-0.16 ± 0.32	0.081 ± 0.62	-0.44 ± 0.69	2.52 ± 0.24
RCOOH O/W (%)	pH 4.6	26.5	93.6	80.2	94.7	52.9	36.9	-
	pH 6.0	1.4	36.6	13.9	41.6	4.3	2.3	-
Polar Surface Area (PSA, Å ²)		61.1	42.0	67.0	67.0	61.0	61.1	44.6
Number of hydrogen bond donors (HBD)		2	1	2	2	2	2	1

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Table II. Chromatographic data for tested products. Delivery was 0.8 ml.min⁻¹.

Product	Mobile phase		Column		Detection UV (nm)	Retention Time (min)
	Water + TFA 0.1%	CH ₃ CN	Supelco Discovery	Merck Chromolith		
SA	50	50	+		210	8.31
BA	50	50	+		229	6.6
3-OHBA	70	30	+		212	8.1
4-OHBA	70	30	+		255	7.8
5-CISA	65	35		+	212	3.5
5-FSA	60	40		+	233	4.3
MeSA	50	50	+		210	10.6

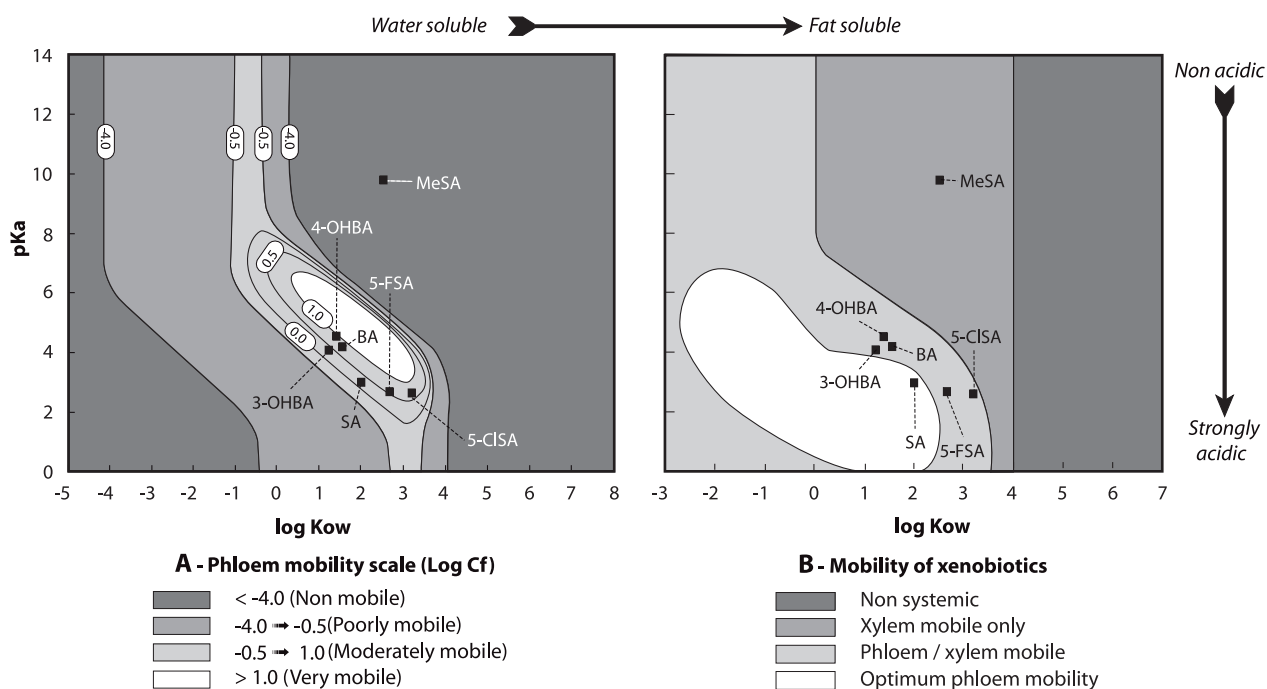


Figure 1. Prediction of phloem mobility of SA, 5-CISA, 5-FSA, BA, 3-OHBA, 4-OHBA and MeSA. (A), Kleier map (Log Cf as a function of Log Kow and pKa) according to Kleier et al (1996); plant parameters are for a short plant. (B), Bromilow model (degrees of mobility as a function of Log Kow and pKa) according to Bromilow et al (1991) .

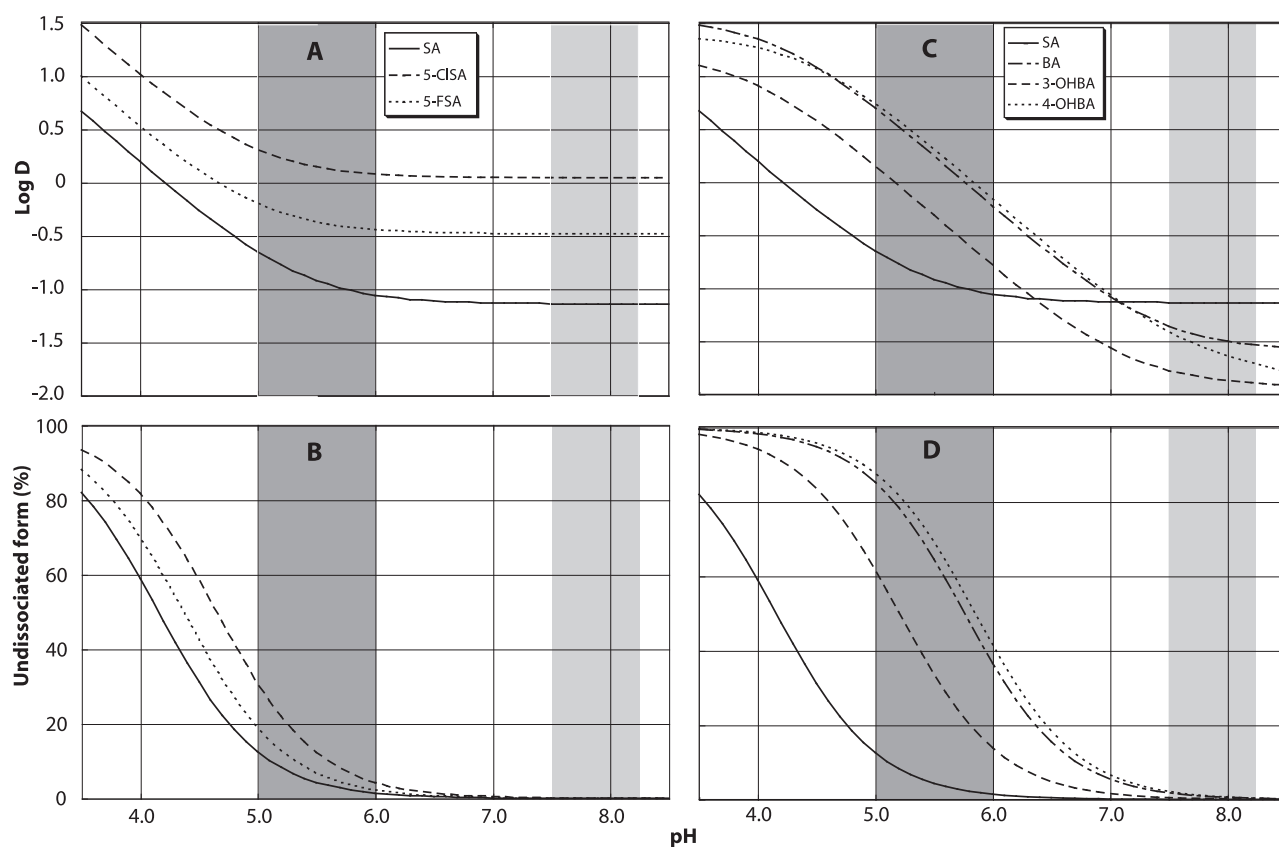


Figure 2. Physicochemical properties of SA and SA derivatives. LogD of SA, 5-CISA, 5-FSA (A) and SA, BA, 3-OHBA, 4-OHBA (C) and percentage of undissociated form of SA, 5-CISA, 5-FSA (B) and SA, BA, 3-OHBA, 4-OHBA (D) in an octanol/water mixture as a function of pH. The apoplast and phloem sap pH values are dash grey and light grey respectively. Note that Δ LogD variations between pH 5.0 and 8.0 are small for SA and halogen derivatives (about 0.5 and 0.25 unit respectively) and high for BA, 3-OHBA, 4-OHBA (about 2.5 units). The apoplastic pH values are from (Giaquinta, 1977) but more acidic values are possible in phloem apoplast taking into consideration the high expression of the PM H^+ -ATPase in phloem cells, especially in companion cells (Bouche-Pillon et al., 1994). SA LogD values and percentage of undissociated form calculated by ACD LogD v 11.01 are similar to those calculated by version 9.0 (Rocher et al., 2006).

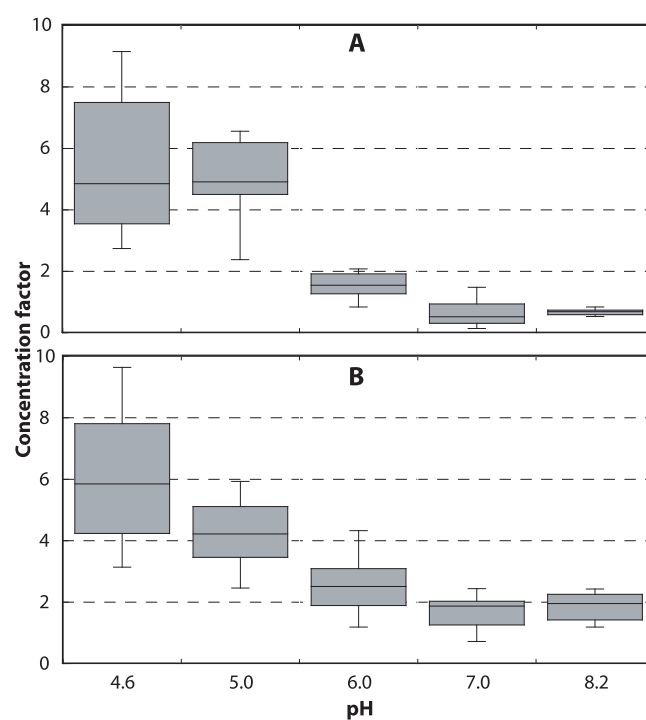


Figure 3. Phloem mobility of SA halogen derivatives. Concentration factor of 5-CISA (A) and 5-FSA (B) (added to the incubation solution at 10 μ M, final concentration) in phloem sap of *Ricinus* as a function of the pH of the incubation medium. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [5-CISA or 5-FSA]_{sap} / [5-CISA or 5-FSA]_{medium}. Boxplots, 10 \leq n \leq 17.

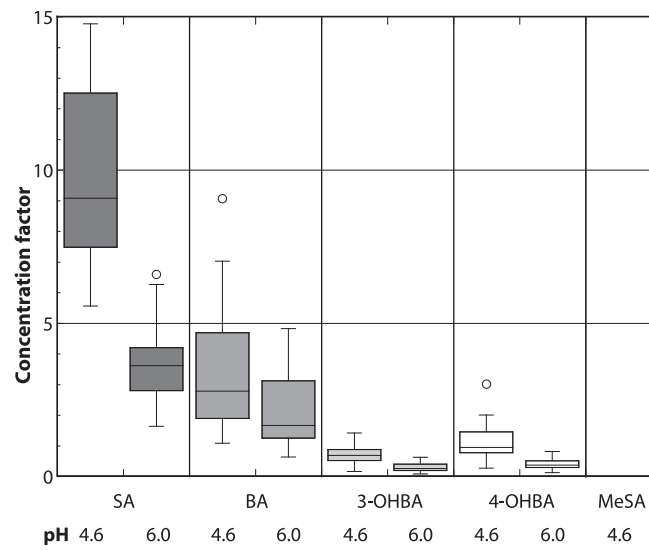


Figure 4. Phloem mobility of SA and SA analogs. Concentration factor of SA, BA, 3-OHBA, 4-OHBA and MeSA (added to the incubation medium at 10 μ M, final concentration) in phloem sap of *Ricinus*. Cotyledons were incubated in a buffered solution at pH 4.6 or 6.0. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [studied product]_{sap} / [studied product]_{incubation medium}. MeSA could not be detected. Box plots, 16<n<29.

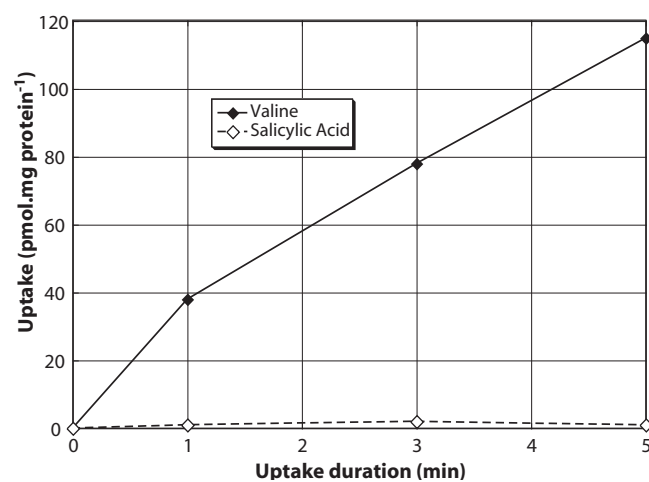


Figure 5. Time dependent uptake of 100 μM [^3H]-Valine and 50 μM [^{14}C]-salicylic acid by yeast strain JT-16 expressing the amino acid transporter ANT1 from *Arabidopsis thaliana* (Chen et al., 2001). Data are the mean of 4 replicates and are expressed as the difference between uptake in JT-16/ANT1 and JT-16/pYES2 (empty plasmid) to measure transport due to ANT1.

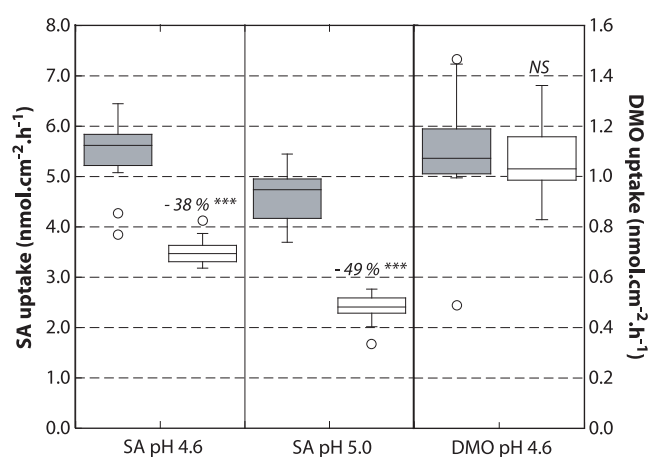


Figure 6. Effect of 1 mM pCMBS on 10 μ M SA or 10 μ M DMO uptake by discs from *Ricinus* cotyledons. Discs were pre-incubated in a buffered solution (pH 4.6 or pH 5.0) for 30 min and then transferred to the incubation medium for 1 h (same solution containing [¹⁴C]-SA or [¹⁴C]-DMO without [grey boxes] or with [white boxes] pCMBS). The Mann-Whitney U test was used to assess statistically significant differences (*** $p < 0.001$; NS not significant). Boxplots, $10 < n < 16$.

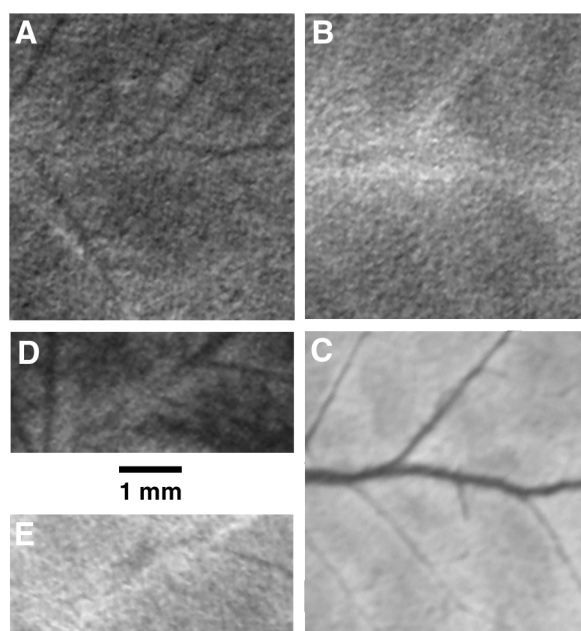


Figure 7. Autoradiographic pictures of the lower side of *Ricinus* cotyledons. Discs were incubated for 1 h in a buffered solution (pH 5.0) containing either 10 μM [^{14}C]-SA (A and B; exposure time 15 h) or 100 μM [^{14}C]-sucrose (D and E; exposure time 51 h) without (A and D) or with (B and E) 1 mM pCMBS. C was a photography of the major veins of the cotyledon area autoradiographed in B. Radioactivity appears in black.

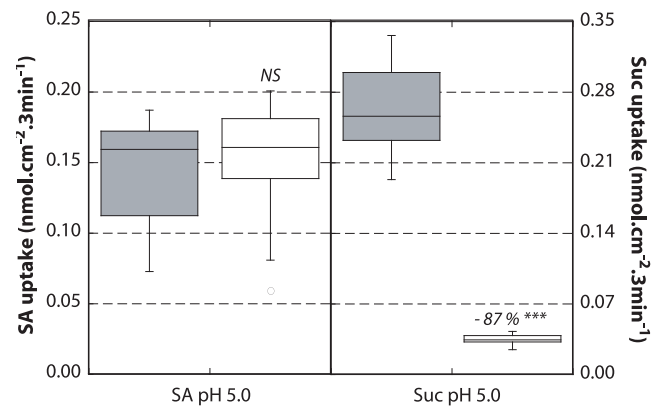


Figure 8. Effect of 1 mM pCMBS on 10 μ M SA or 100 μ M sucrose uptake by discs from *Ricinus* cotyledons for 3 min. Discs were floated on a buffered standard solution (pH 5.0) for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (same solution containing either 10 μ M [14 C]-SA or 100 μ M [14 C]-sucrose without [grey boxes] or with [white boxes] pCMBS) for 3 min. The Mann-Whitney U test was used to assess statistically significant differences (***) $p < 0.001$; NS not significant). Boxplots, $n = 10$.

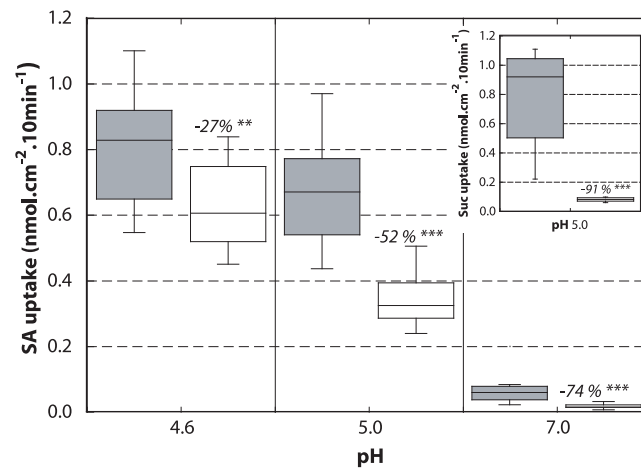


Figure 9. Effect of 1 mM pCMBS on 10 μ M SA uptake or 100 μ M sucrose uptake (insert) by discs from *Ricinus* cotyledons for 10 min. Discs were pre-incubated in a buffered standard solution for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (same solution containing 10 μ M [14 C]-SA or 100 μ M [14 C]-sucrose without [grey boxes] or with [white boxes] pCMBS) for 10 min. The Mann-Whitney U test was used to assess statistically significant differences (*** $p < 0.001$; ** $p < 0.01$). Boxplots, $12 < n < 16$.

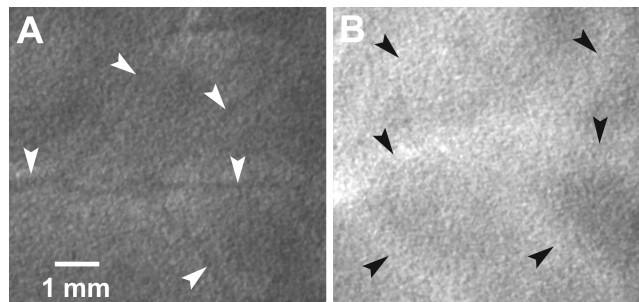


Figure 10. Autoradiographic pictures of the lower side of *Ricinus* cotyledons. Discs were floated for 30 min on a standard preincubation medium and then on the same medium for an additional 15 min without (A) or with (B) 1 mM pCMBS. After the pretreatment, tissues were incubated for 10 min in the same buffered solution (pH 5.0) containing 10 μ M [14 C]-SA without (A) or with (B) 1 mM pCMBS. Exposure time was 100 h. Radioactivity appears in black. Major veins are underlined by arrowheads.