Salicylic Acid, an Ambimobile Molecule Exhibiting a High Ability to Accumulate in the Phloem[1]

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The ability of exogenous salicylic acid (SA) to accumulate in castor bean (*Ricinus communis*) phloem was evaluated by HPLC and liquid scintillation spectrometry analyses of phloem sap collected from the severed apical part of seedlings. Time-course experiments indicated that SA was transported to the root system via the phloem and redistributed upward in small amounts via the xylem. This helps to explain the peculiarities of SA distribution within the plant in response to biotic stress and exogenous SA application. Phloem loading of SA at 1, 10, or 100 μM was dependent on the pH of the cotyledon incubating solution, and accumulation in the phloem sap was the highest (about 10-fold) at the most acidic pH values tested (pH 4.6 and 5.0). As in animal cells, SA uptake still occurred at pH values close to neutrality (i.e. when SA is only in its dissociated form according to the calculations made by ACD LogD suite software). The analog 3,5-dichlorosalicylic acid, which is predicted to be nonmobile according to the models of Bromilow and Kleier, also moved in the sieve tubes. These discrepancies and other data may give rise to the hypothesis of a possible involvement of a pH-dependent carrier system translocating aromatic monocarboxylic acids in addition to the ion-trap mechanism.

The potential of plants to react to pathogens by activating local and long-distance mechanisms has been known for a long time (Chester, 1933). The systemic response was called systemic acquired resistance (SAR) and was explained by the production of a signal released from mature infected leaves and translocated to the upper parts of the plant (Ross, 1966). Then grafting and stem-girdling experiments have suggested that the SAR signal moves in the phloem (Jenns and Kuc, 1979; Guedes et al., 1980). Interest in the role of salicylic acid (SA) in disease resistance arose from the observation that application of exogenous SA or acetylsalicylic acid (aspirin) induces resistance to the *Tobacco mosaic virus* (TMV) in tobacco (*Nicotiana tabacum*; White, 1979) and is highly effective in activating pathogenesis-related (PR) genes (White et al., 1987). A few years later, it was shown that development of SAR in a cultivar of tobacco resistant to TMV is accompanied by a dramatic increase in the level of endogenous SA in the infected leaves after TMV inoculation and also, to a lesser extent, in uninfected upper leaves. Induction of *PR* gene expression parallels the rise in SA levels in both infected and uninfected tissues (Malamy et al., 1990). Furthermore, inoculation of mature cucumber (*Cucumis sativus*) leaves with either the *Tobacco necrosis virus* or the fungal pathogen *Colletotrichum lagenarium* leads to a clear rise in SA levels in the phloem sap and to the development of SAR (Métraux et al., 1990). TMV infection also induces an increase in SA concentration in the phloem sap of tobacco (Yalpani et al., 1991). These data indicate that SA plays an important role in plant defense against pathogen attack and suggest that it may function as an endogenous signal in the transmission of SAR. That SA plays a role in disease resistance was further supported using transgenic tobacco plants expressing the bacterial *nahG* gene encoding salicylate hydrolase, which degrades SA into cathecol. These plants are unable to accumulate SA and to express SAR (Gaffney et al., 1993).

SA phloem transport from the inoculated leaves to the systemically protected tissues is at this time clearly demonstrated. The first strong evidence has come from in vivo labeling with $^{18}O_2$ of the SA synthesized in TMV-inoculated lower leaves of tobacco. Spatial and temporal distribution of [^{18}O]SA indicated that about 70% of the SA detected in the upper uninoculated leaves was ^{18}O-labeled and had therefore been transported from the TMV-inoculated tissue (Shulaev et al., 1995). The biosynthesis and transport of [^{14}C]SA have been studied after injection of ^{14}C-labeled benzoic acid into cucumber cotyledons inoculated with *Tobacco necrosis virus*. Labeled SA has been detected in the phloem and in the upper uninoculated leaf before the development of SAR (Mölders et al., 1996). The specific activity of [^{14}C]SA decreased in the systemically protected tissue, indicating that, in addition to transport,

[1] This work was supported by the Conseil Interprofessionnel du Vin de Bordeaux, the Centre Technique Interprofessionnel de la Vigne et du Vin, the Office National Interprofessionnel des Vins, the Comité Interprofessionnel du Vin de Champagne, and Inter Rhône.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.082537.

the upper leaf also produced more SA. This systemic SA synthesis is likely to be induced by a previous signal (Mölders et al., 1996). Finally, recent data have suggested that the pattern of SAR induction within the Arabidopsis (*Arabidopsis thaliana*) rosette was not confined to the pattern of phloem allocation of [^{14}C]SA from a donor leaf (Kiefer and Slusarenko, 2003). All these data have been discussed in relation to the complexity of the systemic signaling (Métraux, 2001; Durrant and Dong, 2004), a debate initiated in the last decade (Rasmussen et al., 1991; Vernooij et al., 1994; Ryals et al., 1996; Durner et al., 1997; Van Loon, 1997). In this regard, detaching inoculated leaves, as well as grafting experiments, has indicated that SA is not the primary systemic signal (Rasmussen et al., 1991; Vernooij et al., 1994). This one might be a lipid-base molecule (Maldonado et al., 2002). Consequently, SA is now considered as an essential secondary signal for both local resistance and SAR (Maldonado et al., 2002; Durrant and Dong, 2004). In addition, SA, regarded as a plant hormone, has been reported to inhibit seed germination and growth, block the wound response, and reverse the effects of abscisic acid (Shettel and Balke, 1983; Davies, 2004).

Membrane transport of SA in plant cells, unlike in animal cells (Enerson and Drewes, 2003), is very poorly documented. According to Kleier's predicting mathematical model (Yalpani et al., 1991), the physical properties of SA, in terms of pK_a value and the octanol/water partitioning coefficient (log K_{ow}), are nearly ideal for phloem systemicity by way of the ion-trap mechanism. Physiological data about SA uptake properties are restricted to Lemna fronds and isolated cells. Uptake of [^{14}C]SA in *Lemna gibba* is linear for at least 24 h. Because of probable sequestration of both free and bound SA in the vacuole, transfer of SA from mother fronds to daughter fronds cannot be observed (Ben-Tal and Cleland, 1982). SA uptake by tobacco cells is pH dependent and inversely correlated with the increase of medium pH, whereas SA release is likely to involve a Ca-dependent pathway (Chen and Kuc, 1999). Paradoxically, membrane transport of SA metabolites is better known. The vacuolar uptake of SA 2-*O*-β-D-glucoside occurs through an ATP-binding cassette transporter mechanism (Dean and Mills, 2004) and an H^+-antiporter mechanism (Dean et al., 2005).

The purpose of this work was to assess, using the Ricinus system, the capacity of the phloem to load SA from the apoplast in comparison with various endogenous molecules and xenobiotics and to examine whether long-distance transport of SA along the axis is limited or not to the phloem tissue.

RESULTS AND DISCUSSION

Predicting Phloem Mobility of SA in Comparison with Other Moderately Lipophilic Acidic Compounds

Two models based on physicochemical properties of molecules, more precisely their lipophilicity (assessed as the 1-octanol/water partition coefficient log K_{ow}) and their pK_a values are currently used to predict the systemicity of xenobiotics and natural ionizable compounds (Kleier, 1988, 1994, 1998; Bromilow et al., 1991). Much of the data on xenobiotic transport fit rather well into these schemes. These two models were used in this article to predict the phloem mobility of SA as compared with some other natural molecules and xenobiotics (Figs. 1 and 2). It has already been mentioned that measured physicochemical properties of SA (pK_a = 2.98; log K_{ow} = 2.26; Minnick and Kilpatrick, 1939; Hansch and Anderson, 1967) make it well suited for long-distance phloem transport (Yalpani et al., 1991). These values are practically identical to those calculated using ACD LogD version 9.0 software (pK_a = 3.01; log K_{ow} = 2.06). According to the calculations made by this software, two acidic derivatives of fenpiclonil (compounds 2a and 2b) recently synthesized (Chollet et al., 2004, 2005) and 2,4-dichlorophenoxyacetic acid (2,4-D) exhibited a phloem mobility ability near to that of SA (Fig. 2, A and B). In contrast, 3,5-dichlorobenzoic acid (3,5-ClBA; pK_a = 3.46; log K_{ow} = 3.92) was on the boundary between the poorly mobile and the nonmobile molecule areas in both models, whereas 3,5-dichlorosalicylic acid (3,5-ClSA; pK_a = 1.99; log K_{ow} = 4.40) was in the nonmobile molecule area whatever the predicting model used (Fig. 2, A and B).

Endogenous SA Levels in the Phloem and Xylem Saps Exuded from Severed Seedlings

To evaluate the potential ability of phloem to trap exogenous SA from the incubation medium, it was necessary to measure endogenous SA levels in the phloem sap exported by Ricinus cotyledons beforehand. Endogenous SA concentration did not exceed the basal level (<1 μM). Very low values (≈ 0.5 μM) were also noted in the phloem sap of cucumber (control set; Métraux et al., 1990). Endogenous SA concentration in the xylem sap exuded from the Ricinus root system was so low that it could not be detected. These data suggest that SA levels do not change significantly in response to wounding, consistent with previous data (Malamy et al., 1990).

Time-Course Experiments

The Ricinus system is a biological model widely employed to study the phloem uptake of nutrients (Schobert and Komor, 1989; Orlich and Komor, 1992; Zhong et al., 1998) to identify endogenous molecules moving in sieve tubes (Schobert et al., 1995; Antognoni et al., 1998) and to evaluate phloem systemicity of xenobiotics (Bromilow et al., 1987; Delétage-Grandon et al., 2001). Because the castor bean is a symplastic-apoplastic loader (Orlich and Komor, 1992), exogenous SA molecules found in the phloem sap may be taken up from the phloem apoplast or may come, via the symplastic route, from other cells and, especially, from the cotyledon

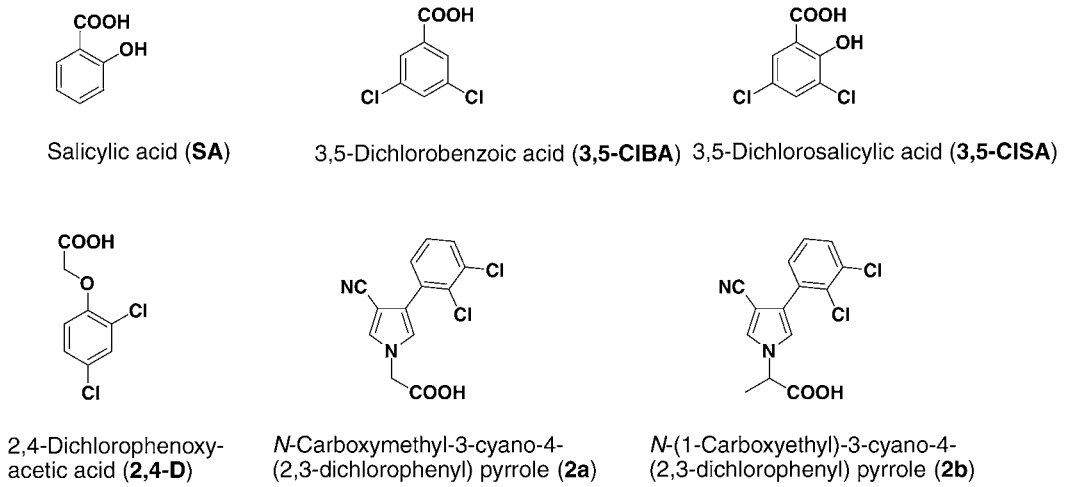

Figure 1. Chemical structure of SA, dichlorinated analogs, and other acidic compounds used in this work.

epidermis. However, in the latter case, exogenous SA molecules must also cross the plasma membrane. In this article, the term SA phloem loading does not discriminate between the various possible sites of transmembrane SA uptake.

When cotyledons were incubated in an acidic solution (pH 4.6) in the presence of SA at 10 μM, the molecule quickly accumulated in the phloem sap. Its concentration increased sharply for about 1.5 h before reaching a near plateau and was then about 10-fold that of the incubation medium (Fig. 3). Thus, in later experiments (pH dependence of SA phloem loading), the sap was collected when SA levels plateaued (i.e. from 2 to 4 h after the beginning of cotyledon incubation). The ability of phloem to accumulate exogenous SA is discussed below.

The presence of exogenous SA in the xylem sap collected from the basal part of the hypocotyl was also

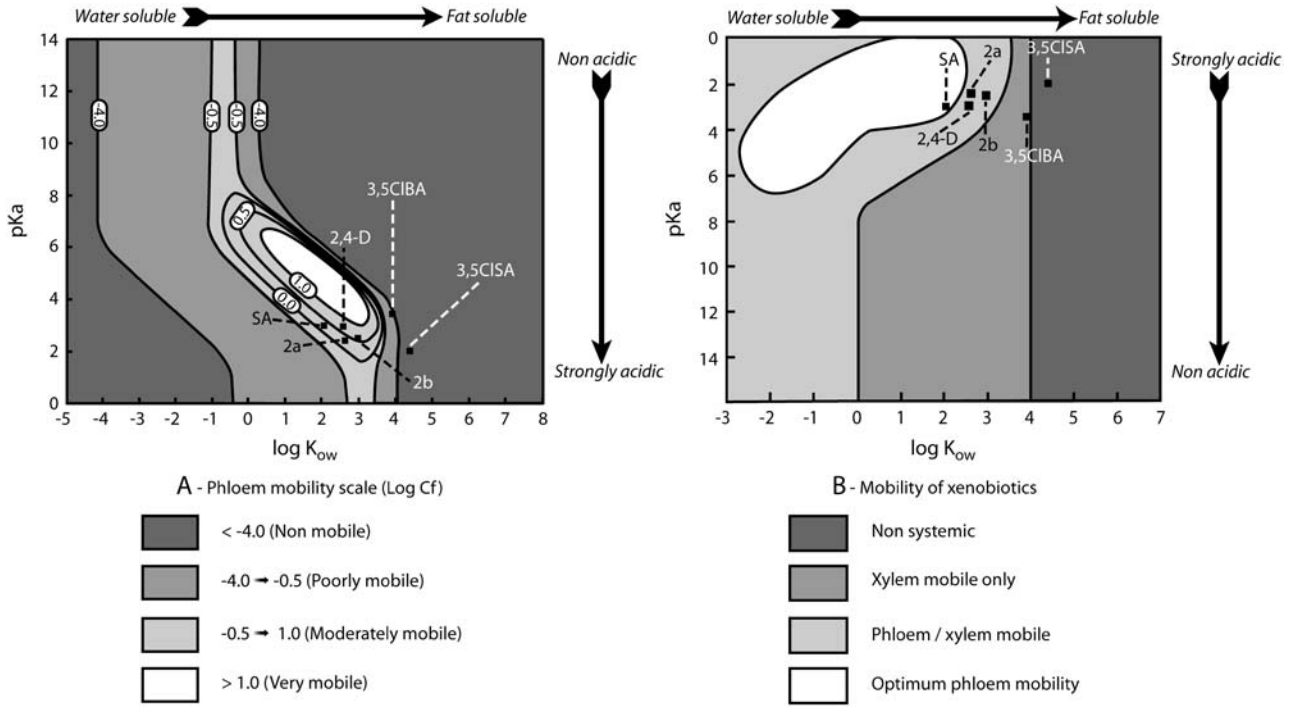

Figure 2. Prediction of phloem mobility of SA, 3,5-ClBA, 3,5-ClSA, and 2,4-D. A, Kleier map (log C_f as a function of log K_{ow} and pK_a) according to Kleier (1994, 1998); plant parameters are for a short plant (Kleier, 1994). B, Bromilow model (degrees of mobility as a function of log K_{ow} and pK_a). For comparison, the predicted phloem mobility of two fenpiclonil acidic derivatives (compounds 2a and 2b; Chollet et al., 2005) was added. Log K_{ow} and pK_a were calculated using ACD LogD suite version 9.0 software.

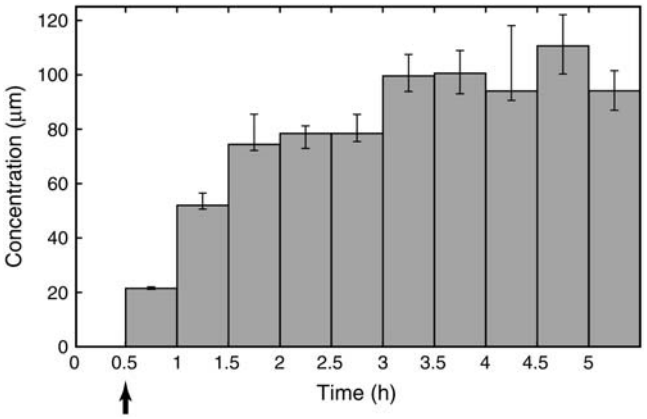

Figure 3. Time course of SA concentration in phloem sap of Ricinus. At time 0, SA at 10 μM (final concentration) was added to the buffered medium, pH 4.6. The hypocotyl was severed in the hook region at time 0.5 h (arrow), and then the sap was collected every half hour during 5 h. The width of the columns indicates the duration of the successive phloem sap collections from the same cut. Medians ± quartiles; $n = 8$ plants.

investigated. In this case, the hypocotyl was severed at different times according to the sets, as indicated in Figure 4. Phloem sap exuded by leaf pressure from the apical part of the seedling and xylem sap exuded by root pressure from the basal part were collected in parallel to compare the time course of SA enrichment in both saps. Preliminary assays indicated that SA concentration in xylem sap was so low during the first hours of transport that it could not be quantified by HPLC. Therefore, experiments were conducted using [^{14}C]SA at 10 μM and the amounts of labeled molecules in both saps were analyzed by liquid-scintillation spectrometry. The time course of labeled molecule enrichment in phloem sap (Fig. 4A) was exactly similar to that of nonlabeled SA (Fig. 3), suggesting that practically all the systemic labeled molecules were unchanged SA. This is consistent with previous data. SA 2-*O*-β-D-glucoside, which accumulates in the tissues in response to an increase of free SA level, does not move in the phloem (Enyedi et al., 1992) but is stored in the vacuole (Dean and Mills, 2004; Dean et al., 2005). Small amounts of labeled molecules were found in xylem sap (Fig. 4B). At first, xylem labeling increased slowly contrary to phloem enrichment. Thus, when the hook was severed 1.5 h after the beginning of cotyledon incubation, apoplastic sap labeling was only 20% of the maximal value noted later (Fig. 4B), compared to 75% for phloem sap (Fig. 4A). Then xylem sap labeling accelerates somewhat before reaching a plateau 4 h after the beginning of [^{14}C]SA uptake. Labeled molecule concentration ($0.67 + 0.14 - 0.20$ μM, median ± interquartiles, $n = 6$ triplicates) in xylem sap exuded by root pressure was then about one-fifteenth that of cotyledon incubation medium and one-one hundred fiftieth that noted in phloem sap (compare Fig. 4, A and B). The velocity of xylem sap exudation (41.5 μL/triplicate/20 min) being 5 times higher than that of phloem sap (12.75 μL/triplicate/30 min), the amount of labeled molecules exuded from the vessels per time unit was about one-thirtieth that released from the sieve tubes under our experimental conditions.

To specify the nature of the labeled molecules moving within the vessels, a complementary experiment was conducted using an incubation medium with unlabeled SA at 100 μM (i.e. a concentration 10 times higher than in the preceding conditions). Four hours after the beginning of SA uptake by cotyledon tissues (i.e. when the amount of exogenous molecules moving in the vessels plateaued), three xylem sap collections of 20 min each were done successively from the same cut and then analyzed by HPLC. SA concentration in the first droplet was $5.92 + 1.61 - 1.66$ μM (median ± interquartile, $n = 4$ triplicates) and therefore about 9 times higher than that of labeled molecules measured in the preceding experiment (Fig. 4B), indicating that most, if not all, of the latter are unchanged SA molecules. It remained the same in the second xylem sap droplet ($5.48 + 1.21 - 1.14$ μM) and then had a tendency

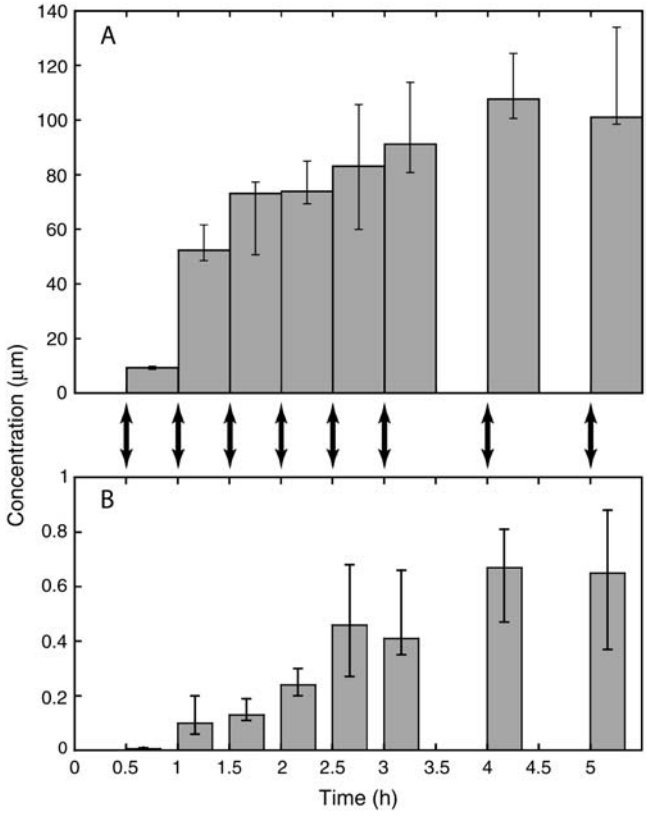

Figure 4. Time course of labeled molecule concentration in phloem sap (A) and xylem sap (B) of Ricinus. Cotyledons were incubated in a buffered solution, pH 4.6, containing [^{14}C]SA at 10 μM concentrations. Seedlings were divided into eight sets and the hook was severed at different times (from 0.5–5 h) according to the sets (double arrows). The phloem sap and the xylem sap ($CaCl_2$ 1 M treatment) were collected for 30 and 20 min, respectively. The width of the columns indicates the duration of phloem and xylem sap collections. Medians ± quartiles; $n = 6$ triplicates.

Table I. *Concentration of some natural compounds and xenobiotics in the Ricinus phloem sap related to the percentage of the undissociated form and log D in two phloem compartments, the phloem apoplast (pH 5.0) and the phloem sap (pH 7.5)*

The sap was collected during the third and fourth hours of incubation (pH 5.0). Mean ± SE; $n \geq 10$.

Compounds	Molecular Weight	RCOOH (%)		Log *D*		Incubation Medium	Phloem Sap
		pH 5.0	pH 7.5	pH 5.0	pH 7.5		
						μM	μM
SA	138.12	9.9	0.0	−0.70	−1.09	1	11 ± 1
						10	79 ± 8
						100	689 ± 60
3,5-ClBA	191.01	97.2	10.0	2.38	0.82	10	24 ± 4
						100	275 ± 39
3,5-ClSA	207.01	12.6	0.1	1.31	1.25	10	6.3 ± 1
2,4-D	221.04	77.5	1.1	0.57	−1.10	10	96 ± 6
						100	700 ± 10[a]
[^{14}C]Glyphosate	169.07	0	0	−5.90	−6.78	100	18 ± 1[a]
N-Carboxymethyl-3-cyano-4-(2,3-dichlorophenyl) pyrrole (2a)	295.12	52.3	0.3	0.10	−1.10	100	10 ± 1.5
						250	42 ± 7[b]
N-(1-Carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl) pyrrole (2b)	309.15	73.4	0.9	0.53	−0.74	100	33 ± 3[c]
[^{14}C]Suc	342.30			−3.48	−3.48	100	2,136 ± 168
[^{14}C]Phe	165.19			−1.39	−1.39	100	2,192 ± 27

[a]From Delétage-Grandon et al. (2001). [b]From Chollet et al. (2004). [c]From Chollet et al. (2005).

to decrease (4.16 ± 0.55 μM in the third droplet). This shows that SA xylem re-exportation remains unchanging for more than half hour despite the cessation of SA phloem transport toward the basal part of the seedling. Treatment of the basal hypocotyl cut with 1 M $CaCl_2$, which induced an intense callose synthesis in sieve-tube pores and cell plasmodesmata, was a severe stress. Therefore, SA levels measured in xylem sap may be somewhat underestimated. On the other hand, without treatment, the amount of SA in the xylem sap (18.3 + 10.3 − 5.7 μM, median ± interquartiles, $n = 11$) was about 3 times higher than those mentioned above, but in this case phloem contamination cannot be excluded.

Our data help to explain why labeled molecule distribution is not limited to the young leaves situated directly above the [^{14}C]SA infiltrated leaf as should be the case according to the phloem allocation pattern (Kiefer and Slusarenko, 2003). They may also suggest

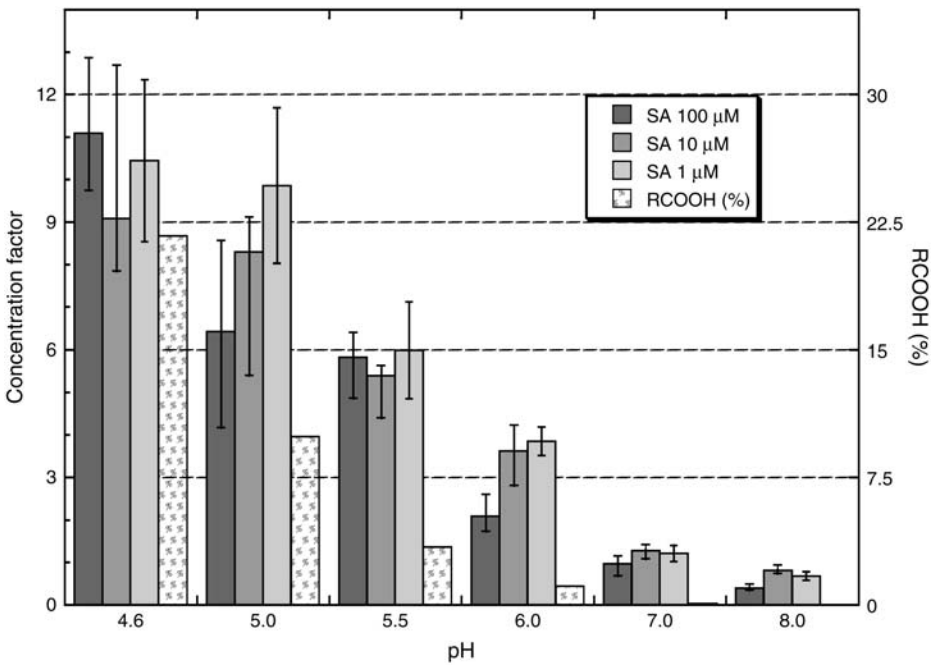

Figure 5. Concentration factor of SA (added to the incubation solution at 1, 10, 100 μM, final concentration) in phloem sap of Ricinus and percentage of SA undissociated form as a function of the pH of the incubation medium. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio $[SA]_{sap}/[SA]_{medium}$. RCOOH (%) was calculated using ACD LogD suite version 9.0 software. Medians ± quartiles; $8 \leq n \leq 12$.

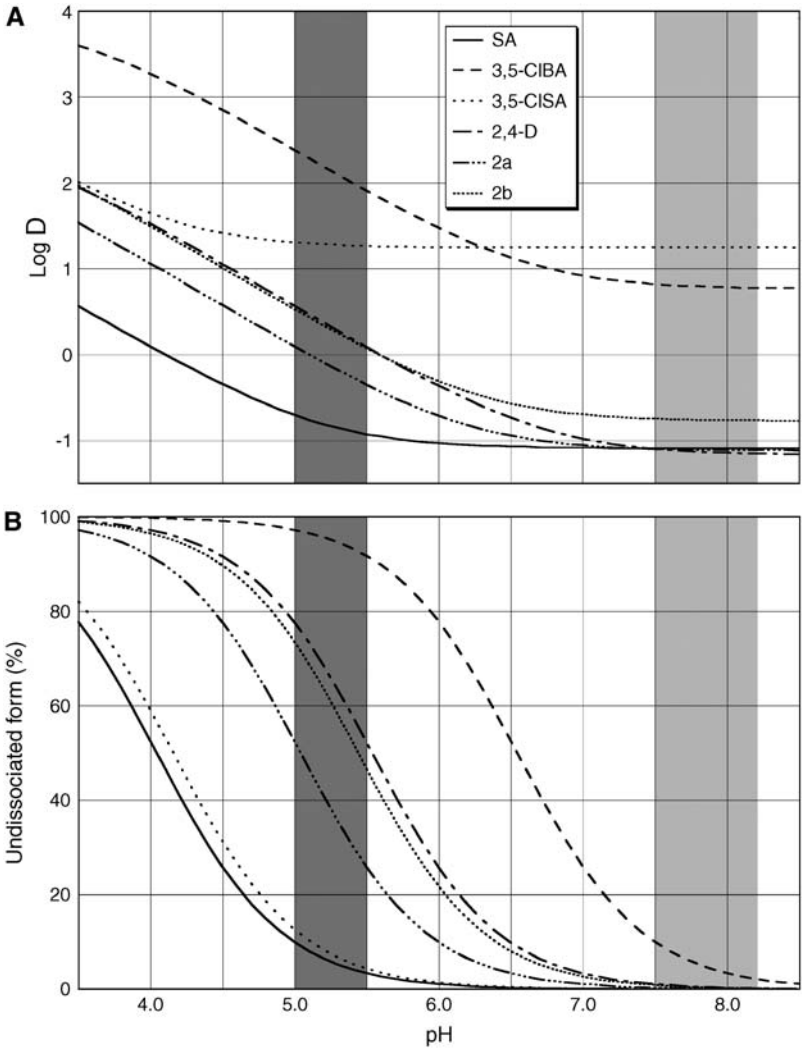

Figure 6. Log *D* (A) and percentage of undissociated form (B) of SA and other monocarboxylic compounds tested as a function of pH. Results were computed using ACD LogD suite version 9.0 software.

that xylem can contribute, although very slightly, to SA enrichment noted in the apical part of plants after mature leaf infection (Shulaev et al., 1995) in addition to phloem allocation (Métraux et al., 1990; Yalpani et al., 1991) and systemic SA synthesis in response to a previous signal (Rasmussen et al., 1991; Meuwly et al., 1995; Mölders et al., 1996). This suggestion is valid only if SA concentrations measured in the Ricinus system can be compared with those observed in response to a pathogen attack. From the very few phloem sap analyses made after leaf tissue inoculation, endogenous SA levels in phloem sap of infected plants range between about 10 to 500 μM, depending on the pathogen (Métraux et al., 1990; Rasmussen et al., 1991). These values are similar to those reported in this study (Fig. 3; Table I). Thus, it can be speculated that endogenous SA levels in xylem sap should be far from negligible in infected plants in case of strong responses induced by a pathogen. If this point is checked, the question of which must be considered is then the one concerning the physiological significance of the apoplastic component of SA long-distance transport, bearing in mind that abscisic acid plays a central role in root-to-shoot signaling via the xylem sap at micromolar concentrations in response to drought stress (Trejo et al., 1995; Jeannette et al., 1999; Assmann, 2004).

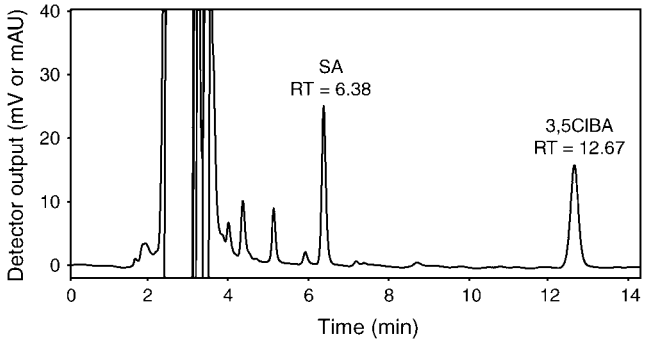

Figure 7. HPLC profile of Ricinus phloem sap. Cotyledons were incubated in a buffered solution, pH 4.6, containing 10 μM SA and 100 μM 3,5-ClBA. Note that endogenous compounds were eluted from 2 to 6 min.

Table II. *Chromatographic data for tested products*

Product	Mobile Phase		Column		Delivery	Detection UV	Retention Time
	Water + TFA 0.1%	CH_3CN	Supelco Discovery	Merck Chromolith			
					mL min^{-1}	*nm*	*min*
SA	50	50	+		0.8	236	6.38
3,5-ClBA	50	50	+		0.8	236	12.67
3,5-ClBA	50	50		+	0.8	211	4.03
3,5-ClSA	53	47		+	0.8	216	4.76
2,4-D	50	50	+		0.8	202	11
2a	57	43		+	0.8	218	5.15
CF	59	41	+		0.8	225	10.48; 11.44

pH Dependence of SA Phloem Loading

SA levels in the phloem sap were dependent on the pH of the incubation medium whatever the SA concentration in this medium (1, 10, or 100 μM). Higher concentrations (1 mM) could not be used because of their toxic effect. The concentration factor in the phloem sap was the highest (about 10-fold) at the most acidic value tested (pH 4.6) and the least (from 0.4–0.8-fold) at pH 8.2 (Fig. 5). A residual SA uptake at pH 7.5 and 8.5 was also observed in tobacco cell suspension cultures (Chen and Kuc, 1999). SA phloem loading was not clearly related to the percentage of the undissociated form of the molecule at the external side of the plasma membrane, as calculated with ACD LogD software. For instance, from pH 4.6 to pH 6.0, the concentration factor in phloem sap decreased from 10 to about 3, whereas the undissociated SA level dropped by 20 times and became marginal (about 1%) at the latter pH value. At pH 7.0, SA accumulated slightly in the phloem (concentration factor = 1.0–1.4), although the molecule was only under its hydrophilic dissociated form (i.e. the nonpermeant form through the phospholipidic layer (Fig. 6B; Table I). The discrepancy between SA uptake and percentage of the undissociated form of the molecule at biological pH is still more marked when a homogeneous aqueous medium is taken into consideration ($pK_a \approx 3.0$). By contrast, data from systemicity tests using the Ricinus system indicate that acidic derivatives of the fungicide fenpiclonil (compounds 2a and 2b) are taken up only in their undissociated form in accordance with the ion-trap mechanism (Chollet et al., 2004, 2005).

A pH dependence of SA and analog uptake similar to that observed in Ricinus tissues has been described in animal cells (Takanaga et al., 1994; Tsuji et al., 1994). This was studied in detail using the human adenocarcinoma cell line, Caco-2 cells, which possess intestinal epithelial-like properties. An efficient SA uptake was noted at the pH values (5.5–6.0) measured along the surface of the intestinal villi. Transcellular transport of SA across Caco-2 cells occurs via a pH-dependent and carrier-mediated transport mechanism specific to monocarboxylic acids (Takanaga et al., 1994; Tsuji and Tamai, 1996). These works led to the characterization of a monocarboxylate transporter family in animal cells (Garcia et al., 1994; Enerson and Drewes, 2003). From these data, it can be asked whether the ion-trap mechanism is the sole mechanism involved in SA uptake in plant tissues, particularly in the phloem.

Measurement of SA Accumulation in the Phloem Sap and Comparison with Other Natural Compounds and Xenobiotics

At pH 5.0 (i.e. a pH value close to that of the phloem apoplast), at least in apolastic loaders (Delrot et al.,

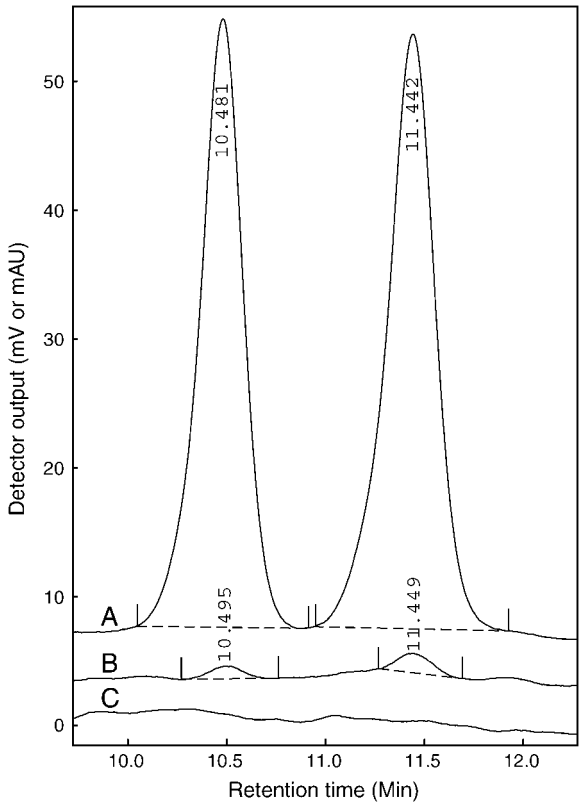

Figure 8. CF as a specific marker for the Ricinus phloem sap. Ricinus cotyledons were incubated for 1 h in a buffered solution, pH 4.6, containing CF at 100 μM before severing the hypocotyl in the hook region. CF was found at 22.6 μM concentration in phloem sap (A), whereas it could not be detected in xylem sap (C). Note that the dual signature of CF was still clear at a very low concentration (0.05 μM; B).

1980), the SA concentration factor in the phloem sap varied from 7- to 11-fold (Table I), although the SA molecule population was predicted to be slightly hydrophilic (Fig. 6A). These values were lower than those noted (21- or 22-fold) for Suc and Phe, which are taken up by specific carrier systems (Lemoine, 2000; Chen et al., 2001), much higher than those reported (0.2- or 0.3-fold) for glyphosate (Delétage-Grandon et al., 2001) and acidic derivatives of fenpiclonil (Chollet et al., 2004, 2005), but close to that noted for 2,4-D, which is less ionized than SA at apoplastic pH (Fig. 6B) but larger in size (Fig. 1). Phloem loading of this phenoxyalkanecarboxylic acid includes two mechanisms, the ion-trap mechanism and a carrier-mediated process (Kasai and Bayer, 1991; Chen et al., 2001).

Unlike the molecules mentioned above, 3,5-ClBA and, especially, 3,5-ClSA remained slightly lipophilic in the Ricinus phloem sap (Fig. 6A), the pH values of which vary from 7.5 to 8.2 according to the stage of development (Hall and Baker, 1972; Vreugdenhil and Koot-Gronsveld, 1988, 1989). This means that these two chlorinated compounds can diffuse back to the apoplastic compartment during long-distance transport. Nevertheless, in contrast to the Kleier and Bromilow model predictions (Fig. 2), 3,5-ClBA was found to be clearly mobile in the phloem (Fig. 7; Table I). Similarly, in contrast to the predictions (Fig. 2), 3,5-ClSA also moved within the sieve tubes (Table I). As already mentioned, the Kleier and Bromilow models give reliable predictions, except for compounds manipulated by a carrier system such as glyphosate (Denis and Delrot, 1993) and carboxyfluorescein (Wright and Oparka, 1994). The concentration factors of 3,5-ClBA (2.5) and 3,5-ClSA (0.6) in the phloem sap are close to those of dichlorinated aromatic conjugates with an α-amino acid function synthesized recently (Rocher, 2004). These latter are translocated by a carrier system (Delétage-Grandon et al., 2001), probably an aromatic and neutral amino acid transporter (Chen et al., 2001). These discrepancies between the predictions (Fig. 2) and experimental data (Table I) may give rise to the hypothesis according to which SA is taken up by a carrier system in addition to the ion-trap mechanism already mentioned (Yalpani et al., 1991). Interestingly, it has been demonstrated recently that biotin, a monocarboxylic acid, is translocated by a Suc carrier (Ludwig et al., 2000). Whatever the mechanism of SA uptake because of the high SA capacity to accumulate in the phloem, early variations in SA concentration in leaf tissue in response to a biotic stress must quickly generate a systemic increase of SA levels in the apical part of the plant. Further work is needed to determine this mechanism.

MATERIALS AND METHODS

Plant Material

Castor bean (*Ricinus communis* L. cv Sanguineus) seeds, obtained from Ball-Ducrettet, were placed in humid cotton wool for 24 h at 27°C ± 1°C prior to sowing in wet vermiculite. Seedlings were grown in a humid atmosphere (80% ± 5%) at 27°C ± 1°C.

Sap Collection and Analysis

Six days after sowing, the endosperm of seedlings (about 20 mm length) was carefully removed (Kallarackal et al., 1989). At this stage of development, the cotyledon cuticle was very thin and permeable to many inorganic and organic solutes (Schobert and Komor, 1989; Orlich and Komor, 1992; Zhong et al., 1998). The cotyledons were then incubated in a buffer solution containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The buffers used were 20 mM MES (pH 5.0, 5.5, and 6.0) and 20 mM HEPES (pH 4.6, 7.0, and 8.0). Buffers containing citrate could not be used at acidic pH due to their chelating effect toward Ca^{2+}. The buffer solution was complemented with SA or another product as described in "Results and Discussion" (Fig. 1; Table I).

At the end of the experiment, the hypocotyl was severed in the hook region at about 2.5 cm below the base of the donor tissues. The phloem and the xylem sap were collected with graded glass microcapillaries from the upper part and the basal part of hypocotyls, respectively. The saps were analyzed immediately or were stored at −80°C until analysis. To prevent exudation from the phloem when collecting the xylem sap, a droplet of 1 M $CaCl_2$ was added to the cut surface for 1 min to plug the sieve tubes (Kallarackal et al., 1989; Antognoni et al., 1998). The surface was then wiped dry with absorbing paper. The purity of the xylem sap collected after the treatment described above was then verified by using 5(6)-carboxyfluorescein (CF), which is known as a symplastic marker (Oparka, 1991). The Ricinus cotyledons were incubated for 1 h in a standard buffered medium, pH 4.6, containing 100 μM CF. After cutting the hypocotyl, xylem and phloem sap were collected after 20 or 30 min, respectively, and then analyzed by HPLC (Table II). Commercial carboxyfluorescein is a mixture of two isomers with a carboxyl group in the 5 or 6 position of fluorescein. Under the chromatographic conditions indicated in Table II, the two isomers could be clearly distinguished (Fig. 8A) and that made the detection of the product easier at very low concentrations (Fig. 8B). CF could not be detected in the xylem sap (Fig. 8C), whereas it was present at 22.6 μM concentration in the phloem sap (Fig. 8A) under our experimental conditions. We also checked that, when applied to the other side of the hypocotyl section, $CaCl_2$ 1 M completely blocked phloem exudation despite the pressure generated by cotyledon tissues.

Saps were analyzed by HPLC after dilution with pure water (1 + 9 and 1 + 1 v/v for phloem sap and xylem sap, respectively). We employed reversed-phase chromatography using a Discovery C16 RP-amide column (length 250 mm, i.d. 4.6 mm; Supelco) or a Chromolith performance RP 18e column (length 100 mm, i.d. 4.6 mm; Merck) in accordance with the procedure set out in Table II. Results were processed with PC 1000 software, version 3.5, from Thermo Electron SA. When radiolabeled molecules were used, phloem sap was analyzed by liquid scintillation spectrometry (TriCarb 1900TR; Packard Instruments).

Chemicals

The compounds to be added to incubation solutions were from Acros Organics (SA, 3,5-ClSA, 4-MES, HEPES, CF) or from Sigma-Aldrich Chimie (3,5-ClBA, SA-carboxy-^{14}C, Suc, Suc-UL-^{14}C).

Physicochemical Properties

Physicochemical properties of SA and other ionizable molecules were predicted using ACD LogD suite version 9.0 software. This unified package of programs calculates log K_{ow} (octanol-water partition coefficient for a neutral species), pK_a (ionization constant in aqueous solution), and log D. The latter is defined as the effective partitioning of all ionic forms of a compound present in equilibrium at a specific pH in octanol-water mixture:

$$\log D = \log\left(\frac{\sum a_i^{org}}{\sum a_i^{H_2O}}\right),$$

where $a_i^{H_2O}$ is the concentration of the ith microspecies in water and a_i^{org} is the concentration of the ith microspecies in the organic phase.

To calculate log D (i.e. the pH-dependent log K_{ow}), the software uses both pK_a and log K_{ow} information. The algorithms for the predictions are based on contributions of separate atoms, structural fragments, and intramolecular

interactions between different fragments. These contributions are derived from internal databases containing experimental data for 18,400 compounds (log K_{ow}) and 16,000 compounds (pK_a), including those for SA, 3,5-ClBA, and 2,4-D. Log *D* is an important parameter considered for bioavailability and absorption studies of drugs (Bös et al., 2001) and agrochemicals (Chollet et al., 2005).

Received May 2, 2006; revised May 2, 2006; accepted June 9, 2006; published June 15, 2006.

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