

Transport of Salicylic Acid in Tobacco Necrosis Virus-Infected Cucumber Plants¹

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The transport of salicylic acid (SA) was studied in cucumber (*Cucumis sativus* L.) using ¹⁴C-labeled benzoic acid that was injected in the cotyledons at the time of inoculation. Primary inoculation with tobacco necrosis virus (TNV) on the cotyledons led to an induction of systemic resistance of the first primary leaf above the cotyledon against *Colletotrichum lagenarium* as early as 3 d after inoculation. [¹⁴C]SA was detected in the phloem or in the first leaf 2 d after TNV inoculation, whereas [¹⁴C]benzoic acid was not detected in the phloem during the first 3 d after TNV inoculation of the cotyledons, indicating phloem transport of [¹⁴C]SA from cotyledon. In leaf 1, the specific activity of [¹⁴C]SA decreased between 1.7 and 8.6 times compared with the cotyledons, indicating that, in addition to transport, leaf 1 also produced more SA. The amount of SA transported after TNV infection of the cotyledon was 9 to 160 times higher than in uninfected control plants. Thus, SA can be transported to leaf 1 before the development of systemic acquired resistance, and SA accumulation in leaf 1 results both from transport from the cotyledon and from synthesis in leaf 1.

Infection of plants by necrotizing pathogens often induces defense mechanisms against subsequent infections by fungi, bacteria, or viruses (Madamanchi and Kuc, 1991; Kessmann et al., 1994). The defense reactions are expressed locally at the site of infection as well as systemically in noninfected parts, and the latter is defined as SAR. The activation of disease resistance in parts of the plant remote from the site of infection implies the translocation of an endogenous signal. A model has been proposed whereby an endogenous signal is produced at the site of primary infection and is translocated through the phloem to other parts of the plant (Jenns and Kuc, 1979; Guedes et al., 1980; Dean and Kuc, 1986a, 1986b).

Several lines of evidence indicate that SA may act as an endogenous signal for SAR. SA applied exogenously to plants induces resistance as well as PR proteins, which typically accompany SAR (White, 1979; Ward et al., 1991). SA is synthesized by plants upon infection. The appearance of SA in the phloem sap and in the upper, noninfected leaves of infected cucumber (*Cucumis sativus* L.) or in the upper, noninfected leaves of infected tobacco or of Arabidopsis correlates with the onset of SAR (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Summer-

matter et al., 1995). The endogenous levels of SA in infected as well as in uninfected leaves of infected plants are sufficient to induce resistance and PR proteins (Yalpani et al., 1991; Enyedi et al., 1992). These results led to a working hypothesis that necrotizing infections trigger the accumulation of endogenous SA, leading to the activation of resistance mechanisms. Support of this hypothesis was provided with temperature-shift experiments using Xanthi-nc tobacco. When grown below 32°C, this cultivar forms necroses upon inoculation with TMV, and SAR, SA, and PR proteins are induced. At or above 32°C, TMV spreads systemically through the plant without necroses but with the formation of typical mosaic-like symptoms of light- and dark-green areas on the leaves. Under these conditions SAR, SA, and PR protein formation are blocked (Yalpani et al., 1991).

Further support for the importance of SA in SAR was obtained using transgenic tobacco and Arabidopsis that were engineered to overexpress SA hydroxylase, an enzyme from *Pseudomonas putida* involved in the metabolism of naphthalene and catalyzing the conversion of SA to the SAR-inactive catechol. Infected transgenic plants are unable to accumulate large amounts of SA and are unable to express SAR (Gaffney et al., 1993; Delaney et al., 1994). When untransformed tobacco scions are grafted onto transgenic rootstocks, SAR and PR-1 expression can be induced in the scion leaves after TMV infection of the rootstock leaves, despite their decreased SA content, suggesting that a signal other than SA can be translocated to the upper leaves and induce resistance (Vernooij et al., 1994). Conversely, when transformed plants are grafted onto wild-type rootstock plants, SAR cannot be observed in the scion leaves after infection of the rootstock leaves. Therefore, SA seems to be necessary for the induction of SAR, but it may not be the primary systemic signal (Vernooij et al., 1994). In cucumber SA is detected in the phloem sap from petioles of upper, noninfected leaves at a time when SA cannot be detected in the phloem that is collected from petioles of the lower, infected leaves. These experiments also suggest that a primary systemic signal exists other than SA, which could induce a systemic accumulation of SA (Rasmussen et al., 1991). Similar leaf detachment assays were performed in TMV-inoculated tobacco. Using sensitive analytical pro-

¹ Funds for this research were provided by grant 31–37098.92 from the Swiss National Science Foundation.

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Abbreviations: BA, benzoic acid; oANI, *ortho*-anisic acid; PR protein, pathogenesis-related protein; SA, salicylic acid; SAR, systemic acquired resistance; TMV, tobacco mosaic virus; TNV, tobacco necrosis virus.

cedures, Shulaev et al. (1995) showed that SAR and PR-1 gene expression could not be detected in the upper leaf unless lower leaves accumulated SA. The possibility that minute amounts of SA translocated from the lower leaves might suffice to induce SAR remains open to discussion, particularly since SA was detected in the phloem of infected cucumber (Métraux et al., 1990) and tobacco (Yalpani et al., 1991).

In tobacco the translocation of SA from an infection site to other parts of the plant was studied using an elegant labeling technique with $^{18}\text{O}_2$ (Shulaev et al., 1995). The results indicate that 69% of the SA detected in the upper leaf was synthesized in the lower infected leaf (Shulaev et al., 1995). In the present study we used ^{14}C -labeled BA injected in cucumber cotyledons to monitor the transport of [^{14}C]SA to the upper leaf after infection. Our results show that, after infection of the cotyledons, the increase in SA observed in the upper leaf results both from synthesis and transport from the infected cotyledon.

MATERIALS AND METHODS

Chemicals

[7- ^{14}C]BA (56 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). oANI was supplied by Sigma.

Plants and Pathogen Inoculations

Cucumber plants (*Cucumis sativus* L, cv Wisconsin SMR-58) were grown as described previously (Meuwly and Métraux, 1993). TNV was prepared from the homogenates of cucumber leaves, as described previously (Métraux and Boller, 1986). The cotyledon of 3-week-old plants was gently rubbed with a cotton swab soaked in the leaf homogenate with or without TNV for mock and TNV inoculation, respectively. To test the resistance, leaf 1 was inoculated with $20 \times 5\text{-}\mu\text{L}$ drops of a spore suspension of *Colletotrichum lagenarium* (200,000 spores/mL). The plants were kept in 100% humidity for 48 h, and necrotic spots were counted 10 d after inoculation (Métraux and Boller, 1986).

Radiolabeling Treatments

Mock- and TNV-inoculated cotyledons were needle-injected in the inoculated area with $20\ \mu\text{L}$ of [7- ^{14}C]BA (100 nCi/ μL) for experiments described in Table I and Fig. 5) or [7- ^{14}C]BA (150 nCi/ μL) for experiments described in Fig. 4). The treated cotyledon and leaf 1 were collected 2 to 4 d later. Experiments with at least three replicate samples each were repeated two to three times with similar results.

Extraction of Plants and Quantitation of Phenolics

Cotyledons and leaves were homogenized in liquid N_2 using a mortar and pestle. oANI was added to the homogenate as an internal standard for the quantitation of free and bound phenolics as previously described (Meuwly and Métraux, 1993). Phloem exudate was collected immediately from the cut surface of the stem that was excised between

cotyledon and leaf 1 as previously described (Métraux et al., 1990). Prior to separation by HPLC, the organic phases were evaporated to dryness and the residues were resuspended in $400\ \mu\text{L}$ of HPLC starting buffer; samples ($30\text{--}200\ \mu\text{L}$, depending on their radioactive content) were taken for analysis. An acetonitrile/phosphate buffer (10 mM, pH 3.5) gradient was run as previously described (Meuwly and Métraux, 1993) at $1.5\ \text{mL min}^{-1}$ and at 35°C on an HPLC system (System Gold; Beckman, Nyon, Switzerland) equipped with a deactivated LC-SAL reversed-phase column ($15\ \text{cm} \times 4.6\ \text{mm} \times 5\ \mu\text{m}$ packing; Supelco, Buchs, Switzerland) preceded by an LC-ABZ guard column ($2.0\ \text{cm} \times 4.6\ \text{mm} \times 5\ \mu\text{m}$ packing, Supelco). SA and oANI were quantified fluorimetrically (spectrophotometer SFM25; Kontron, Zurich, Switzerland) with the wavelengths optimized for each compound (Meuwly and Métraux, 1993). Radioactive phenolic compounds were monitored with an on-line radioactivity detector equipped with a solid yttrium glass detector (Berthold, Regensdorf, Switzerland). The limit of detection was 0.05 nCi.

HPLC Purification and GC-MS Identification of SA

An abundant peak containing material (putative SA) that was both fluorescent and radioactive and that co-eluted with a standard of SA was detected in leaf 1 of cucumber infected and needle-injected with [^{14}C]BA in the cotyledon. This material was collected and further purified using C_{18} reversed-phase HPLC. An acetonitrile/trifluoroacetic acid gradient was run as described below at 35°C on a Beckman HPLC system (see above) equipped with a C_{18} reversed-phase column (ODS, $25\ \text{cm} \times 4.6\ \text{mm} \times 5\ \mu\text{m}$ packing, Supelco) preceded by a C_{18} guard column ($2.0\ \text{cm} \times 4.6\ \text{mm} \times 5\ \mu\text{m}$ packing, Supelco). The column was equilibrated with a mixture composed of trifluoroacetic acid (0.1%) and 20% (v/v) acetonitrile. Elution was programmed at $1\ \text{mL min}^{-1}$, starting with 20% acetonitrile for 3 min and increasing to 60% in 30 min before washing and re-equilibrating periods at 100 and 20%. Radioactivity and fluorescence (excitation at 305 nm, emission at 407 nm) were monitored. Under these conditions the fluorescent and radioactive material co-eluted with a standard of SA at 16 min. The material, corresponding to SA, was collected, evaporated to dryness in vacuo, and applied to a silica gel thin-layer plate (Merck, Darmstadt, Germany). After the plate was developed with a mixture of chloroform and acetic acid (9:1, v/v), only one radioactive band, corresponding to SA, was detected. The radioactive material was eluted with methanol, and the methanol solution was evaporated to dryness.

The purified material was dissolved in $100\ \mu\text{L}$ of methanol, and 1 mL of a solution of diazomethane in ether was added. After 3 h at room temperature the sample was evaporated to dryness and redissolved in $20\ \mu\text{L}$ of chloroform. Identification of methyl-SA was carried by GC (Hewlett-Packard 5890) after separation on a $25\text{-m} \times 0.22\text{-mm} \times 0.25\text{-}\mu\text{m}$ methyl silicone BP5X column (SGE, Melbourne, Australia). GC conditions were: injector 200°C , GC oven held at 100°C for 2 min and programmed at 4°C/min to 290°C , and helium carrier gas at $1\ \text{mL/min}$. Methyl-SA

was characterized by MS (Hewlett-Packard MSD 5970) at an ionization potential of 70 eV and a source temperature of 290°C. The retention time of methyl-SA was 7.8 min with a limit of detection of 50 ng in the scanning mode (50–220 atomic mass units).

RESULTS

Time Course of SAR after Infection with TNV

Figure 1 shows the time course of SAR in leaf 1 of cucumber plants inoculated at d 0 with TNV on the cotyledons. SAR was determined as resistance of leaf 1 against a challenge infection by *C. lagenarium*. SAR can be first detected at d 3 and is maximal 4 d after inoculation of the cotyledon. SAR remained effective throughout the experiment (Fig. 1).

Time Course of SA Production after Infection with TNV

Figure 2 shows the time course of SA accumulation in the cotyledon and leaf 1 of 3-week-old cucumber plants inoculated at d 0 with TNV on the cotyledon. Two days after inoculation there was an increase in free and bound SA (Fig. 2, A and C). Free SA in the cotyledon and in leaf 1 reached a maximum 4 d after TNV infection and then decreased. In control plants the level of SA remained low and steady in the cotyledons but increased somewhat in leaf 1. The reason for the increase in bound SA at d 4 is unclear; it was not observed consistently, as can be seen in Table I. Bound SA continued to increase until the end of the experiment.

Transport of SA after Injection of [¹⁴C]BA

BA was previously shown to be a precursor for SA biosynthesis in infected cucumber plants (Meuwly et al., 1995). The rationale was to use the known precursor of SA in cucumber to label SA in situ to follow the distribution of

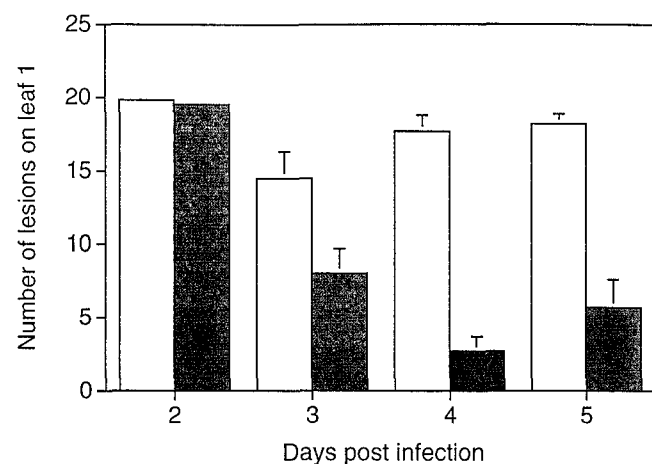


Figure 1. SAR induced by TNV against *C. lagenarium* in cucumber. Leaf 1 was challenged with the fungus between 2 and 5 d after primary inoculation of the cotyledon with TNV (filled bars) or mock inoculation with water (open bars). Results are means (\pm SE) of six replicates.

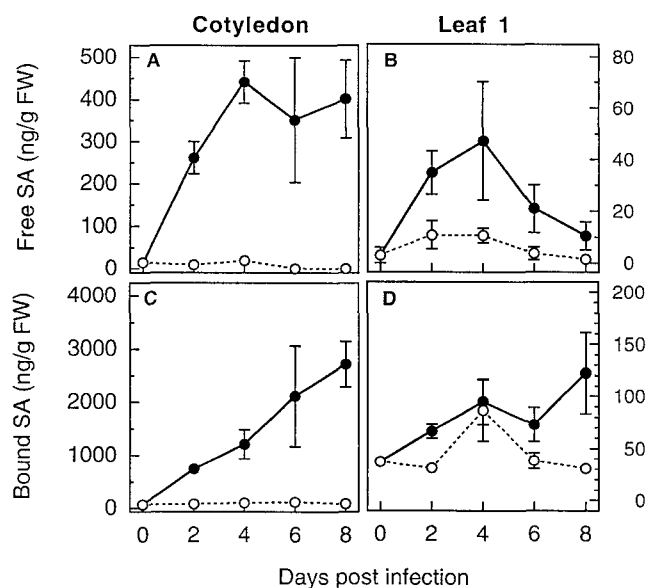


Figure 2. Time course of the increase in SA after infection with TNV. Cotyledons were TNV-inoculated (●) or mock-inoculated (○) at time 0. The cotyledon (A and C) and the first leaf (B and D) were collected at different times after inoculation. Free (A and B) and bound (C and D) SA were measured by HPLC. Results are means (\pm SE) of three replicates. FW, Fresh weight.

SA in a cucumber plant locally infected on the cotyledon. Cells producing radiolabeled SA from BA are just as likely to export labeled SA as unlabeled SA, thus making it possible to monitor SA transport. This also allows one to determine unambiguously the specific activity of SA in the cotyledon and in the leaf. Injection of radiolabeled BA moderately increased the level of unlabeled SA in uninfected cotyledons (Table I; Fig. 2). However, the SA concentration remained much below the levels reached after infection (Table I; Fig. 2). In control cotyledons the injection of radiolabeled BA led to the synthesis of radiolabeled SA, indicating that this conversion can take place even in the absence of infection (Table I). In infected cotyledons the injection of radiolabeled BA led to an increase in unlabeled SA, indicating that SA synthesis is activated in infected tissue and available BA is readily converted to SA (Table I). The absolute concentration of BA injected in the cotyledon, as well as the injection procedure itself, did not have any effect on the induction of SAR (data not shown).

Table I shows a time course of the distribution of [¹⁴C]SA in the infected cotyledon and leaf 1 after injection of [¹⁴C]BA in the cotyledon. Two days after inoculation [¹⁴C]SA was detected in the upper leaf. The specific radioactivity of SA in the cotyledons remained stable throughout the experiment, indicating that the supply of [¹⁴C]BA was sufficient throughout the time course to serve as precursor for SA (Table I). The specific activity in the upper leaves of infected plants was lower than in the cotyledons of the same plants and decreased after 2 d (Table I).

The radioactive material extracted from the leaf eluting at the presumed position of SA was collected and further purified by C₁₈ reversed-phase HPLC, where it co-eluted

Table I. Time course of changes in total SA (ng or nCi) in cotyledon and leaf 1 of cucumber plants after infection of one cotyledon with TNV and injection with [¹⁴C]BA (2 μCi)

Means (±SE) are of five replicates.

Organ	dpi ^a	SA		[¹⁴ C]SA		Specific Radioactivity		Transported SA	
		Control	Infected	Control	Infected	Control	Infected	Control	Infected
		ng/g		nCi/g		nCi/ng		%	
Cotyledon	2	419.8 ± 49.9	3746.1 ± 242.9	121.1 ± 17.6	200.2 ± 21.5	0.2517 ± 0.0136	0.0418 ± 0.0069	–	–
	3	685.4 ± 174.2	5444.8 ± 923.3	290.1 ± 103.8	277.5 ± 37.0	0.3435 ± 0.0505	0.0463 ± 0.0119	–	–
	4	660.9 ± 167.1	5791.3 ± 1023.1	192.6 ± 51.8	204.2 ± 19.6	0.2647 ± 0.0156	0.0321 ± 0.0035	–	–
Leaf 1	2	82.7 ± 7.7	554.9 ± 109.3	3.1 ± 0.9	3.9 ± 0.9	0.0259 ± 0.0068	0.0058 ± 0.0012	10.1 ± 2.7	14.6 ± 3.3
	3	77.7 ± 4.9	804.0 ± 174.4	4.9 ± 1.6	4.4 ± 0.7	0.0491 ± 0.0137	0.0058 ± 0.0014	13.4 ± 2.6	12.8 ± 1.8
	4	74.6 ± 5.5	688.5 ± 116.0	0.7 ± 0.4	2.9 ± 0.4	0.0076 ± 0.0049	0.0043 ± 0.0011	3.3 ± 2.3	13.1 ± 2.6

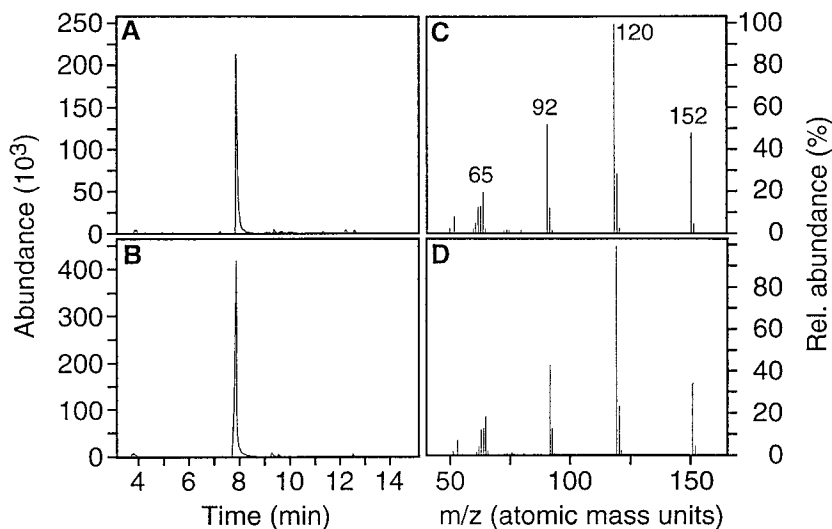
^a dpi, Days postinoculation.

with an authentic standard of SA (data not shown; see "Materials and Methods"). This material was finally purified by preparative TLC and the radioactivity was again found to co-migrate with SA. The purified fluorescent and radioactive material was then esterified with diazomethane. Analysis by GC-MS showed that the methyl derivative eluted at the same retention time as the methyl-SA standard (Fig. 3). The mass spectrum for each sample matched that obtained for the methyl-SA standard, with characteristic mass-ion fragments at *m/z* 152 (*M*⁺), 121, 120, 93, and 92, thus enabling the unambiguous identification of the material as SA in the plants infected with TNV (Fig. 3).

Figure 4 shows that a radiolabeled compound migrating at the position of [¹⁴C]SA can be detected in eluates from phloem exudates collected 1 to 3 d after inoculation of the cotyledons, supporting the notion that SA is translocated from the cotyledon to the leaf. The peak corresponding to [¹⁴C]SA was the only major radioactive material detected in the phloem; it was largest 3 d after TNV inoculation of the cotyledon (Fig. 4, G–I). Three days after TNV inoculation of the cotyledon, free and bound SA were determined using the same method previously described for leaves (Meuwly and Métraux, 1993); they reached levels of 8.5 and 11.5 μM, respectively. In the same phloem exudates the presence of [¹⁴C]BA could not be detected at any time

before the onset of SAR, indicating that the presence of [¹⁴C]SA in the upper leaves is not the result of translocation of [¹⁴C]BA and its subsequent conversion to SA in the leaves (Fig. 4, G–I). The presence of [¹⁴C]BA could not be detected in the homogenates of leaf 1 (data not shown).

The levels of SA transported at various times after TNV inoculation of the cotyledon are represented in Figure 5 and were estimated as follows. If all SA in the upper leaf comes from the cotyledon, the specific activity in leaf 1 should be the same as the activity in the cotyledon. A decrease in the specific activity of leaf 1 reflects dilution caused by both SA present in leaf 1 and SA made upon infection. Since all labeled SA originates from the cotyledon, the amount of unlabeled SA transported to the leaf 1 can be estimated by the ratio of the specific activities in leaf 1 and cotyledon times the amount of unlabeled SA in leaf 1. For example, 2 d after inoculation of the cotyledon with TNV, the level of unlabeled SA, resulting from transport only, is 0.0058 nCi ng⁻¹/0.0148 nCi ng⁻¹ × 554.9 ng g⁻¹ = 76.9 ng g⁻¹, assuming no SA is produced during transport and that SA is not compartmentalized differently from its labeled counterpart in the cotyledon. At each time after inoculation both the values for transported and synthesized SA were calculated as described above for each individual plant described in Table I, and the results are presented in Figure 5.

Figure 3. Identification of SA by GC-MS. Total ion GC traces (A and B) and mass spectra (C and D) of the methyl derivative of the putative SA from leaf 1 of cucumber plants after primary inoculation of the cotyledon with TNV (B and D) and of a methyl-SA standard (A and C), respectively.

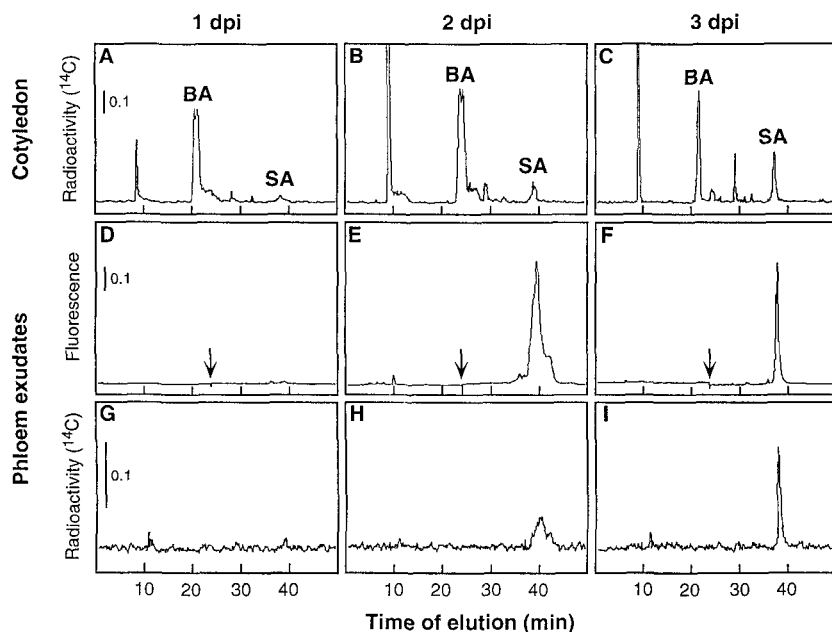


Figure 4. Typical fluorescence (D–F), and radioactivity (A and B and G–I) traces in HPLC chromatograms of acid-hydrolyzed extracts of cotyledon (A and B) and phloem exudates (D–F) from cucumber plants infected with TNV. Cotyledons were infected and needle-injected with 20 μL of [^{14}C]BA (150 nCi/ μL) at time 0. Cotyledons and phloem exudates from 12 different plants were collected and pooled at different times. The arrows in D to F indicate the change in the emission wavelength from 365 to 407 nm; the excitation wavelength remained at 305 during the entire analysis. The scale (bar = 0.1) is identical for each type of detection. dpi, Days postinoculation.

DISCUSSION

Infection of cucumber cotyledons with TNV leads to SAR against *C. lagenarium* and to an increase in the level of SA in the leaf. This system exhibits the same features as the cucumber plants used previously, from which leaf 1 was used for induction and leaf 2 was used for challenge infection (Métraux and Boller, 1986; also reviewed by Madamanchi and Kuc, 1991). Cucumber cotyledons utilize BA as a precursor for the synthesis of SA as was demonstrated for cucumber and tobacco leaves (Yalpani et al., 1993; Meuwly et al., 1995). SA was previously reported in the phloem sap of infected cucumber (Métraux et al., 1990; Rasmussen et al., 1991) as well as in tobacco plants (Malamy et al., 1990; Yalpani et al., 1991), suggesting the transport of SA from

the site of infection to the upper, uninfected leaves. This observation led to the hypothesis that SA might be the long-distance systemic signal for SAR (Malamy et al., 1990; Métraux et al., 1990).

Here we present data confirming the presence of [^{14}C]SA in the cotyledons, phloem, and leaves after injection of [^{14}C]BA in the cotyledons (Table I; Fig. 4). Since no [^{14}C]BA or any radiolabeled substance other than SA was detected in the phloem (Fig. 4) and no [^{14}C]BA was detected in the leaves (data not shown), the results presented here confirm the systemic movement of [^{14}C]SA from the site of synthesis after infection. It is interesting that SA can be detected in the phloem before SAR is measurable in leaf 1 (Figs. 1 and 4), confirming previous observations (Métraux et al., 1990). Two days after inoculation of the cotyledon radiolabeled SA was detected in leaf 1 (Table I). At the same time, 80 ng/g fresh weight SA was transported to leaf 1 (Fig. 5), which represented 14% of the amount present in leaf 1, compared with 10 ng/g fresh weight, which was transported to leaf 1 in control plants. It is likely that these values represent an underestimate, since not all [^{14}C]SA made in the cotyledon can be translocated and part of SA is catabolized.

Rasmussen et al. (1991) did not observe an increase in SA in the phloem of cucumber prior to the development of SAR. This may be explained by the lower sensitivity of TLC used in their study compared with HPLC with on-line fluorescence/radioactivity detection used here. It is likely that the other part of SA measured in leaf 1 results from synthesis in leaf 1, as indicated by the decrease in specific activity of SA after infection (Table I). This is in agreement with a previous report showing systemic synthesis of SA in cucumber after localized infection (Meuwly et al., 1995). Whether this increase in SA results from the activation of the synthesis by SA itself, by a metabolite derived from it, or by another signal remains to be determined.

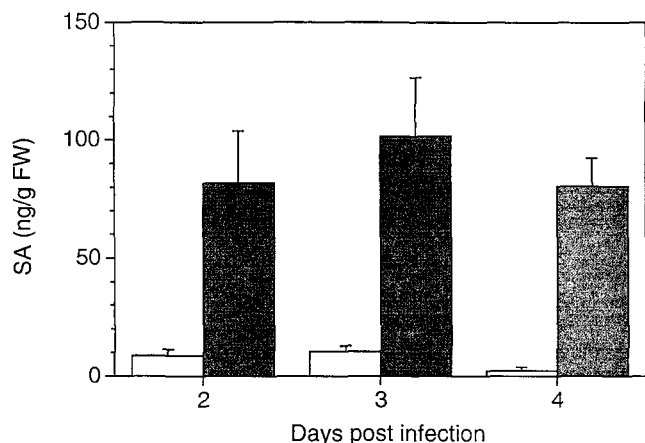


Figure 5. Transported SA in leaf 1 after TNV inoculation of the cotyledon. The values were calculated from the results shown in Table I. For calculation procedures, see the text. TNV, Filled bars; mock inoculation with water, open bars. Results are means (\pm SE) of five replicates. FW, Fresh weight.

The data presented here support observations made in infected tobacco in which ^{18}O labeling was used to show that up to 70% of SA accumulating in the upper leaves arrives from the inoculated leaf (Shulaev et al., 1995). To account for the rest of the SA detected in the upper leaf, transport of BA from the infected leaf and its conversion to SA were proposed by Shulaev et al. (1995). In cucumber, this is unlikely to be the case, since we did not observe transport of BA in the phloem of infected cucumber plants (Fig. 4).

It was shown in tobacco that a small increase in endogenous SA concentration (59%) is sufficient to induce the accumulation of PR protein in the leaves (Yalpani et al., 1991). On the other hand, SAR depends on the presence of a minimal concentration of endogenous SA as demonstrated by the loss of SAR in transgenic tobacco overexpressing salicylate hydroxylase (Vernooij et al., 1994). Generally, both in tobacco and in cucumber, infection leads to only small systemic increases in endogenous SA levels (Yalpani et al., 1991; Meuwly and Métraux, 1993; Mölders et al., 1994), indicating that these plants are likely to be responsive to small changes in endogenous SA. In cucumber the increase in SA resulting from translocation might therefore contribute to the induction of SAR.

In summary, we have demonstrated that in cucumber SA synthesized in infected cotyledons can be translocated to the leaf and that this transport occurs before SAR is detected. The possibility that translocated SA plays a role as a systemic signal inducer in cucumber, therefore, cannot be dismissed.

ACKNOWLEDGMENTS

We wish to thank Dr. Liliane Sticher for her critical reading of this manuscript. The technical assistance of Ghislaine Rigoli is also gratefully acknowledged.

Received February 16, 1996; accepted June 30, 1996.

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