

Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*

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Received: 2 January 2009 / Accepted: 5 April 2009 / Published online: 16 May 2009
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Abstract Salicylic acid (SA) and its glucoside (SAG) were detected in xylem sap of *Brassica napus* by HPLC–MS. Concentrations of SA and SAG in xylem sap from the root and hypocotyl of the plant, and in extracts of shoots above the hypocotyl, increased after infection with the vascular pathogen *Verticillium longisporum*. Both concentrations were correlated with disease severity assessed as the reduction in shoot length. Furthermore, SAG levels in shoot extracts were correlated with the amount of *V. longisporum* DNA in the hypocotyls. Although the concentration of SAG (but not SA) in xylem sap of infected plants gradually declined from 14 to 35 days post infection, SAG levels remained significantly higher than in uninfected plants during the whole experiment. Jasmonic acid (JA) and abscisic acid (ABA) levels in xylem sap were not affected by infection with *V. longisporum*. SA and SAG extend the list of phytohormones potentially transported from root to shoot with the transpiration stream. The physiological relevance of this transport and its contribution to the distribution of SA in plants remain to be elucidated.

Keywords *Brassica napus* · Phytohormones · Salicylic acid · Salicylic acid glucoside · *Verticillium longisporum* · Xylem sap

Abbreviations

ABA	<i>cis</i> -Abscisic acid
Dpi	Days post inoculation
JA	Jasmonic acid
<i>m/z</i>	Mass-to-charge ratio
SA	Salicylic acid
SAG	Salicylic acid glucoside

Introduction

Salicylic acid (SA) is a phytohormone involved in the control of plant defence against pathogens. The role of SA in the activation of systemic acquired resistance (SAR) has been proven on biochemical and genetic levels. Treatment with SA or its analogues enhances the resistance of plants against pathogens, while mutants with disrupted SA synthesis and plants engineered to hydroxylate SA enzymatically exhibit increased susceptibility to diseases (reviewed by Loake and Grant 2007). Conversely, plants expressing bacterial genes for SA synthesis acquired enhanced resistance to pathogen infection (Verberne et al. 2000). While the role of SA in SAR is undisputed, SA is not the primary factor that is translocated from the place of infection to distant organs where it triggers SAR (Rasmussen et al. 1991; Vernooij et al. 1994). The identity of the primary systemic signal has not yet been conclusively established.

The major nonvolatile conjugate of SA is glucoside (SAG). SAG is regarded as a storage form or detoxification product of SA. Accumulation of SAG in the vacuole (Dean et al. 2005), and the absence of SAG from the phloem sap of plants with induced SAR (Enyedi et al. 1992), supported this view. Further corroboration came from the observation of a slow, long-lasting defence-like response in tobacco

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suspension culture treated by SAG, which was explained by a gradual release of SA by SAG hydrolysis (Kawano et al. 2004). This view was challenged recently by the work of Umemura et al. (2008), who used RNAi-mediated silencing of the glucosyl transferase responsible for SAG formation and showed that the activity contributed significantly to SAR development. The authors suggested that glucosylation of SA may actually be a key mediator of SAR. Overexpression of SA glucosyl transferase in *Arabidopsis* led to contradictory results, since the levels of free SA and SAG (as well as the glucose ester of SA) decreased rather than increased (Song et al. 2008).

Salicylic acid derivatives can be transported over long distances principally via phloem or xylem. Phloem moves plant metabolites in the source-to-sink direction while xylem transport takes place along the transpiration stream. Solutes can thus be transported in opposite directions in xylem and phloem, depending on the ratio of photosynthetic and respiratory activities of the organs involved. In phloem, SA levels are elevated upon pathogen attack (Métraux et al. 1990; Yalpani et al. 1991). In cucumber, free SA is translocated by phloem from cotyledon to leaves (Mölders et al. 1996), but phloem transport of SA from an inoculated leaf is low (Rasmussen et al. 1991). Conjugated forms of SA were not found in phloem sap of infected tobacco plants (Enyedi et al. 1992). In contrast to phloem, until recently, only indirect evidence indicated that xylem may be involved in the transport of SA. The distribution of [^{14}C]-SA from an infiltrated leaf within the *Arabidopsis* rosette could not be explained by phloem translocation alone (Kiefer and Slusarenko 2003). Rocher et al. (2006) applied [^{14}C]-labelled SA exogenously to the cotyledons of castor bean and observed movement of radioactivity via phloem towards the basal part of the seedling, where part of it was transported into the xylem and carried back to the shoot. The authors suggested that long-distance transport of SA via xylem may contribute to SA accumulation in distal parts of the plant in response to infection of its lower parts, in addition to endogenous synthesis and allocation by phloem. Their attempts to determine endogenous SA in xylem sap of castor bean seedlings failed.

Verticillium longisporum is a soil-borne fungal pathogen with host specificity for the genus *Brassica*. The fungus enters host plants through their roots and colonises xylem vessels, to which it remains restricted for most its infection phase (Eynck et al. 2007; Zhou et al. 2006). The main motivation for the taxonomical separation of *V. longisporum* from the closely related *V. dahliae* is the ability of *V. longisporum* to infect crucifers. *V. dahliae* is therefore commonly used in studies of *V. longisporum* pathogenicity as a control to test for the specificity of the observed effects.

In this work we demonstrate that SA and SAG are present in xylem sap of *Brassica napus* plants, and show that the concentrations of both compounds increase following infection with *Verticillium longisporum*.

Materials and methods

Plant material

Rapid-cycling rape (*Brassica napus* var. *napus*, genome ACAacc) was originally provided by P.H. Williams (Department of Plant Biology, University of Wisconsin-Madison, WI; Crucifer Genetics Cooperative, Stock number 5). Seeds were surface sterilised by immersing them in 70% ethanol for 30 s and then rinsing with sterilised tap water for 30 s three times.

Fungal isolates

Verticillium longisporum VL 43 from *B. napus* and *V. dahliae* VD 73 from *Linum usitatissimum* were used in this study. Both strains were isolated from plants grown in northern Germany as described in Zeise and von Tiedemann (2001).

Fungal stocks with $1\text{--}3 \times 10^6$ conidia ml^{-1} were stored at -80°C in 25% glycerol.

Preparation of inoculum for plant infection

Shake cultures were started by adding 500 μl spore suspension ($1\text{--}3 \times 10^6$ conidia ml^{-1}) to 300 ml Czapek Dox broth. The inoculated broth was incubated at 23°C on a rotary shaker at 100 rpm in the dark. After 10–14 days, the culture was filtered through sterile gauze. Spore concentration was determined using a haemocytometer and diluted to 1×10^6 spores ml^{-1} .

Plant inoculation and cultivation

Rapid-cycling rapeseeds were germinated in autoclaved sand in climate-controlled chambers (30 kLux, 60% humidity, 22/20°C day/night, and 15-h day length; Philips TL5 HO lamps). Seven-day-old seedlings were inoculated by root-dipping in a spore suspension of either *V. longisporum* (isolate 43, 1×10^6 spores ml^{-1}) or *V. dahliae* (isolate 73, 1×10^6 spores ml^{-1}) for 30–45 min. As a control, seedlings were dipped in sterile water. After inoculation, single seedlings were potted in a sterile sand–soil mixture (1:1) and grown under the conditions described above.

Xylem sap preparation

Rapeseed plants were harvested 28 days post inoculation (dpi). For time-course experiments, xylem sap was collected at 14, 21, 28, and 35 dpi. Shoots were cut above the hypocotyl, and xylem sap was collected by pressurizing roots to 4×10^5 Pa for 15 min using a Scholander pressure chamber (Scholander et al. 1965). Xylem sap was filtered through a 0.2- μ m-Teflon filter (WICOM, Heppenheim, Germany) and subjected to HPLC–MS analysis immediately or stored at -20°C . Xylem sap was checked for contamination with cytoplasm by determining malate dehydrogenase activity (absence of activity indicated absence of cytoplasmic contamination).

Determination of sucrose concentration in xylem sap

The concentration of total sucrose and glucose in xylem sap was determined using the assay kit Enzytec fluid sucrose (r-biopharm, Darmstadt, Germany), which is based on enzymatic hydrolysis of sucrose, phosphorylation of glucose by the activity of hexokinase and oxidation of glucose-6-phosphate to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase. The increase in NADH concentration was monitored by UV absorption at 340 nm (Specord 40, Analytik Jena, Germany). The concentration of free glucose was determined using an Enzytec fluid D-Glucose assay kit (r-biopharm, Darmstadt, Germany), which is based on the same principle except for the omission of the sucrose hydrolysis step. Standard deviation of technical replicas was lower than 4% for both assays. The concentration of sucrose was determined by subtracting the glucose concentration from the sum of sucrose and glucose.

Preparation of shoot extracts

The shoots of plants from which xylem sap was obtained were shock frozen with liquid nitrogen and stored at -80°C . Frozen shoot material was lyophilized (at -20°C) and ground in a ball mill. Extraction was performed as follows: to 50 mg dry shoot material (two replications for each plant), 1.5 ml methanol (gradient quality; Fisher Scientific, Schwerte, Germany) and 1.0 ml chloroform (p.a.; Roth, Karlsruhe, Germany) were added. The mixture was shaken vigorously and incubated for 5 min at 37°C . Subsequently, 2 ml either twice-distilled water or 2% formic acid (puriss. p.a.; Fluka, Seelze, Germany) in water were added. The suspension was shaken for 2 h at room temperature. The samples were centrifuged at 3,583 g for 15 min, the phases were separated, and the polar phase dried under vacuum at 30°C . The residue was dissolved in 1 ml methanol/bi-distilled water (1:9), and the solution was filtered through a 0.2- μ m-Teflon filter. Samples were

directly subjected to HPLC–mass spectrometry (HPLC–MS) or stored at -20°C .

High performance liquid chromatography and mass spectrometry

Methanol (gradient quality; Fisher Scientific, Schwerte, Germany), acetonitrile (gradient quality; VWR, Darmstadt, Germany), and acetic acid (p.a.; Fluka) were used for the mobile phases.

For full-scan analysis, an HPLC system equipped with autosampler (ProStar 410, Varian, Darmstadt, Germany), binary pump system (ProStar 210, Varian), degasser, and a column oven coupled to an ion trap mass spectrometer (500-MS, Varian) via electrospray ionisation source was used. Samples were injected onto a reversed-phase column Polaris C18-Ether (100 \times 2 mm, 3 μ m particle size; Varian) kept at 40°C . The mobile phase consisted of a binary gradient of 7 mM acetic acid in 95% water with 5% acetonitrile (A), and 7 mM acetic acid in methanol (B): 0.0–2.2 min 10% B, 2.2–25.0 min from 10% B to 98% B, 25–30 min 98% B, followed by washing (98% B) and re-equilibration (10% B) steps at a flow rate of 0.2 ml/min. The electrospray ionisation source was operated either with negative or positive spray polarity with the mass spectrometer operating in a full scan mode at a mass range of a mass-to-charge ratio (m/z) 100–1,000 and a scan speed of 5,000 Da/s. Raw data files were converted into NetCDF format and processed using XCMS Analyte Profiling Software (Smith et al. 2006).

Phytohormones, SA, and SAG were quantified using multiple reaction monitoring mode on the HPLC system specified above connected to a triple-quadrupole mass spectrometer (1,200 L, Varian) with an electrospray interface. Standards of SA, jasmonic acid (JA), and abscisic acid (ABA) were purchased from Sigma-Aldrich. Salicylic acid glucoside was kindly provided by H. Kneifel (Research Centre Jülich, Institute Phytosphere, Jülich, Germany). The analytical compounds were separated on a reversed-phase column (Polaris C18-A, 125 \times 2 mm, 5 μ m particle size, Varian) at 40°C and eluted isocratically with 7 mM acetic acid in 47.5% water, 2.5% acetonitrile, and 50% methanol at a flow rate of 0.2 ml/min. The eluate was subjected to negative electrospray ionization, and the ions were detected using the following mass transitions: SAG m/z 298.8 \rightarrow m/z 137 and m/z 298.8 \rightarrow m/z 93; SA m/z 136.8 \rightarrow m/z 93; ABA m/z 262.8 \rightarrow m/z 153, JA m/z 208.9 \rightarrow m/z 59.

Quantification of *V. longisporum* DNA

Fungal biomass was determined in lyophilized hypocotyls from single plants harvested at 28 dpi. DNA was isolated using the cetyltrimethylammonium bromide (CTAB)

method as modified by Brandfass and Karlovsky (2006), except for the final precipitation with polyethylene glycol (polyethylene glycol 6000, Serva, Heidelberg, Germany), which was performed as follows. After chloroform extraction, 700 μl DNA solution in CTAB buffer was transferred to a 1.5-ml tube containing 200 μl polyethylene glycol (30%) and 100 μl 5 M NaCl; the solution was mixed, incubated for 20 min at room temperature, and centrifuged for 15 min at 14,000g. The pellet was washed with 70% (v/v) ethanol, dried, and dissolved in 30 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). DNA concentration was estimated by densitometry after electrophoresis in 0.8% (w/v) agarose gels (Cambrex, Rockland, ME) in TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.5) at 7 V/cm for 60 min. Densitometry was performed as described previously (Brandfass and Karlovsky 2006). Fungal DNA was quantified by real-time PCR as described by Eynck et al. (2007).

Statistics

Data are presented as mean \pm standard deviation. The standard deviations for the increases in SA or SAG relative to the control were calculated according to the error propagation formula. Differences in concentrations of phytohormones and SA derivatives among uninfected, *V. longisporum*-infected, and *V. dahliae*-infected plants were determined by ANOVA, and the significance of correlation coefficients was determined with 1-tailed *t* tests (SPSS 15.0; SPSS, Chicago, IL). All correlation coefficients presented in the figures are significant at the 0.05 level.

Results

Salicylic acid glucoside in xylem sap and shoots of *B. napus*

Xylem sap extracted from *B. napus* plants infected with *V. longisporum* and from healthy controls was analysed by HPLC–MS to identify metabolites affected by the infection. Plants at 28 dpi were used because at this time the amount of xylem sap extractable from a single plant exceeded 100 μl . Xylem sap was filtered and subjected to HPLC–MS analysis directly.

The concentration of the sum of sucrose and glucose in xylem sap was determined in order to estimate the contamination of xylem sap with phloem sap. Xylem sap of infected plants contained 2.17 ± 1.04 mM sucrose plus glucose, and xylem sap of uninfected controls contained 3.10 ± 0.31 mM sucrose plus glucose, indicating that contamination of xylem with phloem sap was lower than 0.3% (see “Discussion” for calculation).

Comparison of the metabolic profiles of infected and healthy plants revealed a number of signals with intensities that were increased in infected plants. One of these signals had $m/z = 299$ —the same as that of the molecular ion of SAG in negative ionisation mode.

Methanol/chloroform/water extracts were prepared from shoots of the plants from which the xylem sap originated. Metabolic profiles of the polar phase of these extracts were recorded in negative ionisation mode and examined for signals with m/z of 299. Three signals were found, the intensity of one of which increased after infection. At the retention time of the metabolite generating this signal (3.5 min), another signal at m/z 137 was detected. Because 138 is the molecular weight of SA and the difference between 299 and 137 corresponds to a glucose residue, we hypothesised that the signal at m/z 299 originated from SAG, and that the ion with m/z of 137 originated from free SA generated during ionisation by in-source fragmentation. The hypothesis was proven correct by comparing the xylem metabolite with m/z 299 with chemically synthesised SAG. Comparison of retention times and analysis of product ion spectra on a triple quadrupole confirmed that the signal with m/z 299, which was enhanced by infection with *V. longisporum*, originated from SAG (Fig. 1). The extracted ion chromatogram for negative ion m/z 299 for xylem sap obtained from plants infected with the taxonomically related species *V. dahliae*, which is not a pathogen for *B. napus*, was indistinguishable from chromatograms generated for noninfected plants (Fig. 1), showing that the increase in SAG concentration was specific for *V. longisporum*.

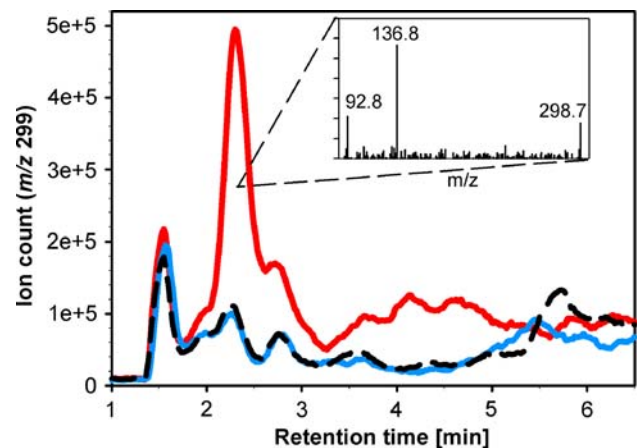


Fig. 1 Selected ion chromatogram of salicylic acid glucoside (SAG) in xylem sap. Xylem sap of healthy plants (black dashed line), *Verticillium longisporum*-infected plants (red line) and *Verticillium dahliae*-infected plants (blue line) was analysed by HPLC–MS in selected ion monitoring mode. Ion chromatograms of SAG (m/z 299, $[\text{M}-\text{H}]^-$) and product ion spectra of m/z 299 are shown (Colour figure online)

Relationship between SA and SAG concentrations, disease symptoms and fungal biomass

Because SAG is formed from SA by glucosyl transferase (Yalpani et al. 1992), in addition to SAG we also determined the concentration of SA in xylem sap and shoot extracts from individual plants. Plants infected with *V. longisporum* and healthy controls were harvested at 28 dpi, xylem sap was obtained from the root and hypocotyl part of each plant, and the shoots were subjected to acidic methanol/chloroform/water extraction. Xylem sap samples and shoot extracts were analysed by HPLC–MS, using multiple reaction monitoring on a triple-quadrupole. Large differences in SA and SAG concentrations among individual infected plants indicated that SA and SAG were affected by the level of infection, which is notoriously variable (Eynck et al. 2007). Infected plants contained 0.2–1.5 μM SAG in xylem sap and 2–189 nmol/g dry weight in shoots. Healthy plants contained only 0.06–0.11 μM SAG in xylem sap and 0.51–1.48 nmol/g dry weight in shoots. The concentrations of SA and its glucoside were correlated strongly both in shoot tissue and xylem sap, the levels of SA being about twice as high as the levels of SAG (Fig. 2a, b). Both SA and SAG levels were also correlated between xylem sap and shoot extracts (data for SAG are shown in Fig. 2c).

As stunting is the most prominent symptom of the infection of rapid-cycling rape with *V. longisporum* in the greenhouse, we investigated the relationships between SA and SAG levels and shoot length. SAG and SA concentrations in both shoot extracts and xylem sap were correlated with a reduction in shoot length (Fig. 3).

Because disease symptoms are caused, directly or indirectly, by fungal colonisation, we determined the amount of fungal DNA in the hypocotyls of individual infected plants at 28 dpi and compared these values to the SAG concentration in the shoot and to shoot length. A strong correlation between SAG levels in the shoot and the amount of *V. longisporum* DNA in hypocotyls was found (Fig. 4a). The reduction in shoot length and the amount of *V. longisporum* DNA were also correlated, although the correlation was less pronounced (Fig. 4b).

Changes in concentrations of SA, SAG, JA, and ABA in xylem sap during infection of *B. napus* with *V. longisporum*

The work of Johansson et al. (2006) hinted at the involvement of JA, and possibly ABA, pathways in the infection of *A. thaliana* with *V. longisporum*. We therefore simultaneously determined the concentrations of SA, SAG, JA and ABA in xylem sap collected from *B. napus* plants at 14, 21, 28, and 35 dpi. Because plants were too small to deliver sufficient amounts of xylem sap at early time

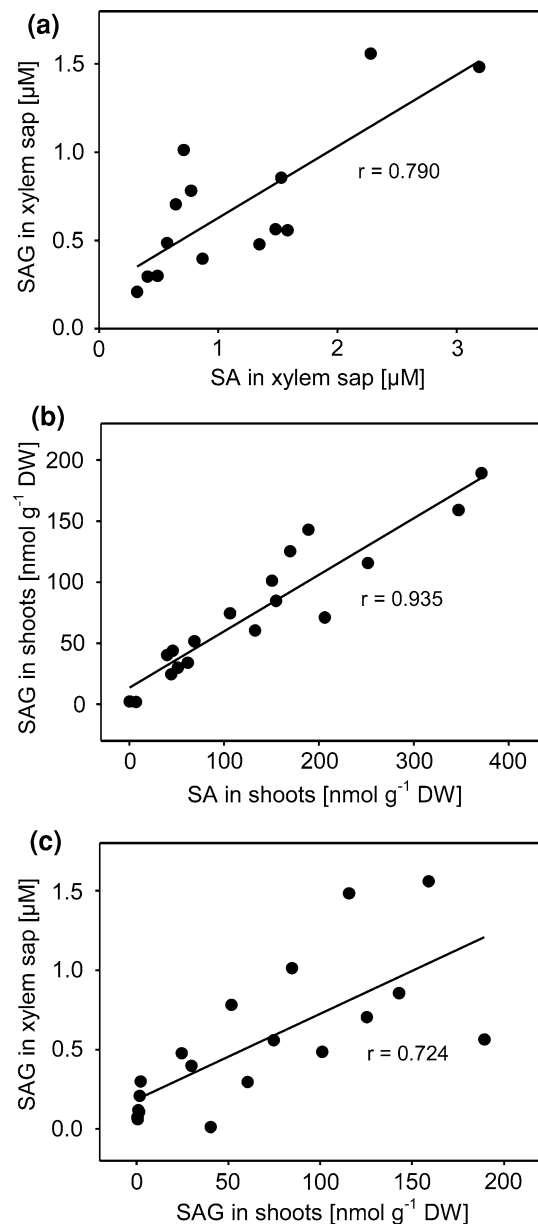


Fig. 2 Comparison of the concentrations of salicylic acid (SA) and SAG in xylem sap and shoot extracts. Xylem sap from *V. longisporum*-infected single plants was analysed 28 days post infection (dpi). Pearson correlation is given (r), all correlations are significant at $P \leq 0.01$ (1-tailed t test). **a** SA versus SAG in xylem sap, **b** SA versus SAG in shoots, **c** SAG in shoots versus SAG in xylem sap

points, samples from four plants were pooled for each analysis. Salicylic acid glucoside, SA, JA, and ABA concentrations were determined by injecting filtered xylem sap directly onto the HPLC column. The results are shown in Fig. 5. For both healthy plants and *V. dahliae*-infected plants, SAG concentrations remained at the same level (20–40 nM) over the course of the experiment. After infection with *V. longisporum*, SAG concentrations

Fig. 3 Concentrations of SAG (a, c) and SA (b, d) in shoot extracts (a, b) and xylem sap (c, d) in relation to shoot length. Xylem sap from *V. longisporum*-infected single plants was analysed 28 dpi. Pearson correlation is given (r), all correlations are significant at $P \leq 0.05$ (1-tailed t test)

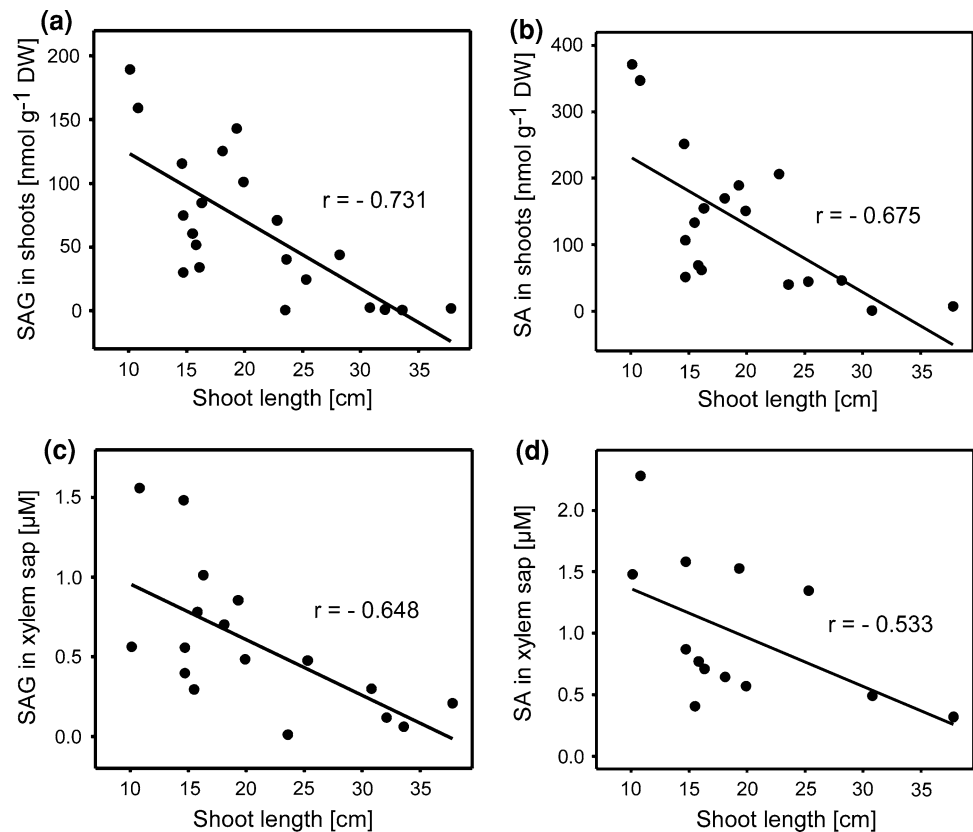
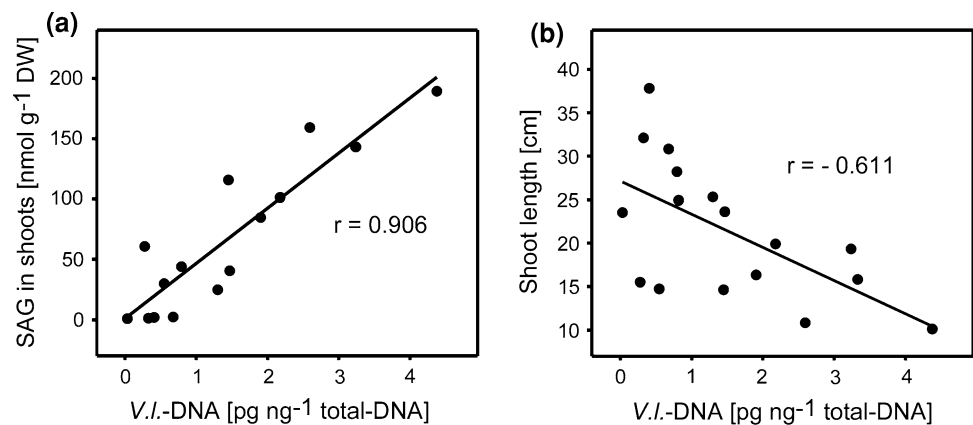


Fig. 4 Concentrations of SAG in shoot extracts (a) and shoot length (b) in relation to the amount of fungal DNA in the hypocotyl. Shoot extracts from individual *V. longisporum*-infected plants were analysed 28 dpi; fungal DNA (*V. l.-DNA*) in hypocotyls was determined by real-time PCR. Pearson correlation is given (r), all correlations are significant at $P \leq 0.01$ (1-tailed t test)



increased strongly at 14 dpi or earlier and declined gradually thereafter, while remaining significantly higher than in the controls throughout the whole experiment. Similarly, SA concentration in xylem sap was significantly higher as compared to uninfected plants at all time points in plants infected with *V. longisporum* but not with *V. dahliae* (Fig. 5a). The ratio of SA concentration in xylem sap of *V. longisporum*-infected versus healthy plants increased from 4 to 9 between 14 and 35 dpi, because the gradual decrease in SA concentration was less pronounced in infected plants than in healthy plants (Fig. 5b).

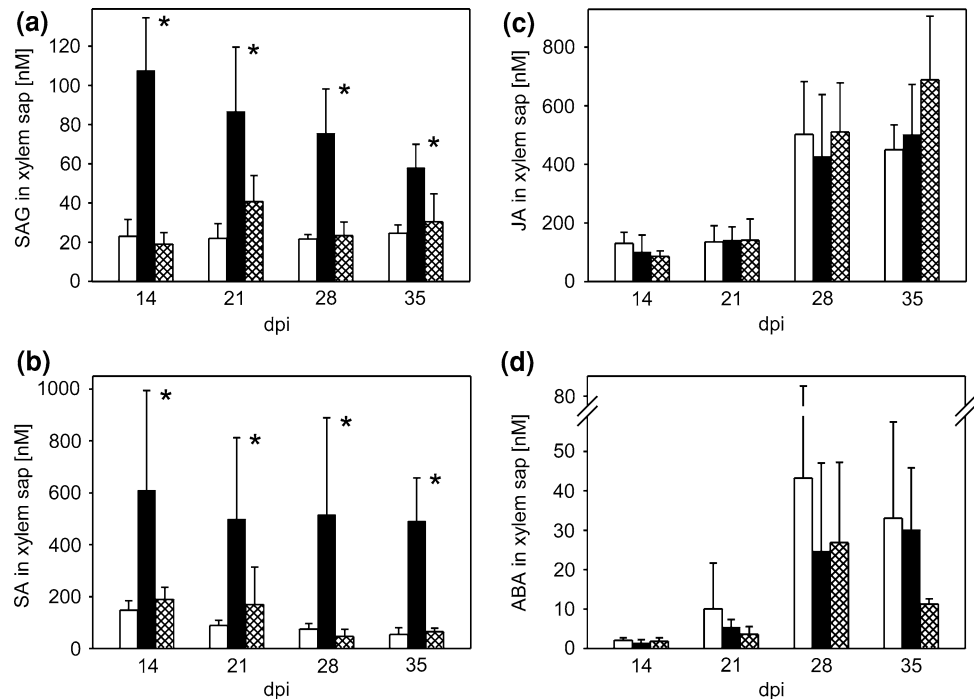
The levels of JA and ABA in xylem sap increased with plant age, but did not differ significantly between

V. longisporum-infected, *V. dahliae*-infected and healthy plants (Fig. 5c, d).

Discussion

We detected SA and SAG in xylem sap of *B. napus* and showed that their concentrations increased after infection with *V. longisporum*. High concentrations of SA occur in phloem sap after pathogen attack (Métraux et al. 1990; Rasmussen et al. 1991) but neither free endogenous SA nor its derivatives have been reported from xylem sap. A recent study of the re-distribution of [¹⁴C]-labelled SA (Rocher

Fig. 5 Concentrations of SAG, SA, jasmonic acid (JA) and abscisic acid (ABA) in xylem sap of *V. longisporum*- and *V. dahliae*-infected *Brassica napus* plants. Analysis of xylem sap from healthy plants (*white*), *V. longisporum*-infected plants (*black*) and *V. dahliae*-infected plants (*hatched*) at different time points after infection is shown. Each data point represents an average of five samples (each sample pooled from four plants). Error bars Standard deviation. * Significant differences ($P \leq 0.05$) between xylem sap samples from *V. longisporum*-infected plants and both remaining samples (mock- and *V. dahliae*-infected). **a** SAG, **b** SA, **c** JA, **d** ABA



et al. 2006) inspired a hypothesis that the transpiration stream contributes to long-distance transport of SA in plants, but an attempt to detect SA in xylem sap by these latter authors failed.

In principle, the SA and SAG found in xylem sap could have originated from contamination with other tissues. Pollution with cytoplasm from damaged cells appeared negligible because activity of malate dehydrogenase was undetectable, but contamination with phloem sap cannot be excluded. Sucrose is a biomarker for phloem; while present in only trace quantities in xylem sap (Nakamura et al. 2008), sucrose concentrations of 0.98 and 1.34 M were reported for phloem sap of *B. napus* (Lohaus and Moellers 2000). Comparison of these values with our results (2.17 ± 1.04 mM of sucrose plus glucose for infected plants, 3.10 ± 0.31 mM for uninfected controls) indicates that xylem sap contained less than 0.3% phloem sap. The concentration of free glucose in xylem sap was 1.31 ± 0.53 mM (controls) and 1.30 ± 0.08 mM (infected plants), while Nakamura et al. (2008) reported 0.30 mM. Since we assumed that all glucose in xylem originated from hydrolysed sucrose, the contamination of xylem sap with phloem sap was probably even lower than the estimate given above.

Salicylic acid glucoside is formed by glucosyl transferase in a concentration-dependent manner (Dean et al. 2005). Because glycosylation is an intracellular process, SAG must be transported into xylem from the surrounding tissues. Similar ratios of SAG and SA in shoot tissue and in xylem sap (Fig. 2a, b) indicate that both metabolites are

secreted into xylem with the same efficiency, contrasting with the selective transport of SAG into the vacuole (Dean and Mills 2004). Recent findings that the suppression of SA glycosylation in rice by gene silencing reduced the development of resistance (Umemura et al. 2008) indicates that SAG may play a central role in SAR. Overexpression of SA glucosyl transferase in *Arabidopsis*, however, increased susceptibility to a bacterial pathogen (Song et al. 2008). This contradiction was resolved by finding that SA and SAG in transgenic plants decreased rather than increased due to increased activity of SA methyl transferase (Song et al. 2008).

Many biologically active secondary metabolites synthesised in the roots are transported acropetally with the transpiration stream (Satoh 2006). Abscisic acid is synthesised during drought stress in roots and transported to the shoots, where it triggers a drought response (Hartung et al. 2002). Zeatins are also carried from roots to shoots (Kato et al. 2002; Kuroha et al. 2005). According to our data, in *B. napus*, SA and SAG are transported in the same manner. Our results proved Rocher et al. (2006) right in their prediction that “endogenous SA levels in xylem sap should be far from negligible in infected plants”. In this context, it is interesting to note that the specific activity of SA glucosyl transferase in oat was ten-times higher in roots than in shoots (Yalpani et al. 1992).

In *A. thaliana*, the role of the SA pathway in response to *Verticillium* infection has been studied by Veronese et al. (2003), Tjamos et al. (2005) and Johansson et al. (2006). The first two groups worked with *V. dahliae* and obtained

contradictory results regarding the expression of SA-dependent pathogenesis-related genes. Because the taxonomical status of *V. longisporum* is controversial, and its separation from *V. dahliae* is sometimes difficult, one of the strains used in these studies might actually have been *V. longisporum* (A. von Tiedemann, unpublished results). The third laboratory worked with *V. longisporum* and described the induction of PR-1 and PR-2 as early as 7 dpi, but the SA pathway was found not to be involved in the defence because plants with defective SA pathway did not exhibit enhanced susceptibility. In our experiments, the amount of SAG in the shoot was correlated with fungal biomass in the hypocotyl (Fig. 4a). Similar correlations were found for SA in shoot extracts and fungal DNA, and for SA and SAG in xylem sap and DNA (data not shown). Furthermore, both SA and SAG concentrations were correlated negatively with shoot length (Fig. 3). The determination of SA and SAG concentrations in *A. thaliana* after infection might help assess the suitability of the *A. thaliana/V. longisporum* pathosystem as a model for *Verticillium* disease of oilseed rape.

V. dahliae-infected plants were indistinguishable from uninfected controls both in SA and SAG levels and in their phenotype (Fig. 5a, b). The lack of disease symptoms in *B. napus* infected with *V. dahliae* has been demonstrated before by Eynck et al. (2007).

Johansson et al. (2006) demonstrated that the JA pathway was involved in the response of *A. thaliana* to *V. longisporum*, and that ABA was not required (although an *ABA2* mutant was impaired in defence reaction). In our work with *B. napus*, JA levels in xylem sap increased, and those of SAG decreased with plant age (Fig. 5). This is indicative of an antagonistic relationship between the two pathways (Takahashi et al. 2004). Because infected and healthy plants exhibited similar changes in JA concentration over time, however, aging-related processes were more likely the cause. JA is known to be involved in senescence (Fukushige et al. 2002). Obviously the roles of JA in the defence of *Arabidopsis* and *Brassica* against *V. longisporum* are different, possibly reflecting the fact that *Brassica*, unlike *Arabidopsis*, is a natural host for the fungus.

Veronese et al. (2003) showed that ABA (but not SA or JA) acted as a regulator of the *A. thaliana* response to *V. dahliae* infection (the strain used might actually have been *V. longisporum*). Antagonism between the ABA and SA pathways was demonstrated in *Arabidopsis* (Yasuda et al. 2008), but in our system the increase in SA concentration caused by *V. longisporum* infection did not affect endogenous ABA levels (Fig. 5).

This work demonstrates for the first time the presence of SA and SAG in xylem sap. These two metabolites thus expand the list of phytohormones transported with the transpiration stream from roots to shoots (Satoh 2006). The

biological role of the enhanced levels of SA and SAG in xylem sap after infection with a vascular pathogen remains to be elucidated.

Acknowledgments This research work was part of an inter-facultative research unit FOR546 funded by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany. We gratefully thank Prof. H. Kneifel (Forschungszentrum Jülich, Jülich, Germany) for providing us with the SAG standard. The technical assistance of P. Bartoschek and M. Bossmann is highly appreciated.

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