Systemin in *Solanum nigrum*. The Tomato-Homologous Polypeptide Does Not Mediate Direct Defense Responses\(^1\)\(^{[W]}\)

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We extend Ryan's seminal work on the 18-amino acid polypeptide systemin in tomato's (*Solanum lycopersicum*) systemic wound response to the closely related solanaceous species *Solanum nigrum*. We compared wild-type plants to plants transformed with an inverted repeat prosystemin construct (IRSys) to silence the expression of the endogenous *S. nigrum* prosystemin gene. In wild-type plants elicited with wounding + oral secretions from *Manduca sexta* larvae, trypsin-proteinase inhibitors (TPIs) accumulated even though prosystemin transcripts were down-regulated. Neither reducing the endogenous systemin levels by RNAi nor complementing the plants with systemin by exogenously supplying the polypeptide through excised stems significantly increased TPI activity, indicating that systemin and TPIs are not correlated in *S. nigrum*. The performance of two herbivore species from two feeding guilds, *M. sexta* larvae and *Myzus persicae nicotianae*, did not differ between wild-type and IRSys plants, demonstrating that varying endogenous systemin levels do not alter the direct defenses of *S. nigrum*. Field experiments with wild-type and IRSys plants and the flea beetle *Epitrix pubescens* supported these greenhouse data. That levels of oral secretion-elicited jasmonic acid did not differ between wild-type and IRSys plants suggests that systemin is unlikely to mediate jasmonate signaling in *S. nigrum* as it does in tomato. We conclude that the tomato-homologous polypeptide does not mediate direct defense responses in *S. nigrum*.

Plants not only respond locally to leaf damage caused by wounding, herbivory, or pathogen attack, but also induce defenses in distal, unwounded leaves. These systemic defense responses have been extensively studied in tomato (*Solanum lycopersicum*), where an 18-amino acid polypeptide called systemin is known to play an essential role in generating the mobile wound signal. Systemin is processed from its larger precursor, prosystemin, which is synthesized and processed in the vascular phloem parenchyma cells (Narvaez-Vasquez and Ryan, 2004). Constitutive prosystemin mRNA expression has been found throughout the plant except for the roots (McGurl et al., 1992). After leaf wounding, prosystemin mRNA is induced systemically; the highest accumulation is seen after 3 to 4 h (McGurl et al., 1992). Like the prosystemin mRNA, proteinase inhibitor (PI) I mRNA, which encodes for a protein with antinutritional effects against several lepidopteran herbivores (Johnson et al., 1989; Ryan, 1990), accumulates systemically after wounding; it is most abundant 8 to 10 h after wounding (McGurl et al., 1992). Young tomato plants supplied with low concentrations of systemin through their cut stems accumulated PI I and II (Pearce et al., 1991). This positive correlation between systemin and PIs is supported by the work of McGurl et al. (1992), who transformed tomato plants with an antisense prosystemin construct. A transgenic line lacking prosystemin was almost completely suppressed in its systemic induction of PIs I and II. Furthermore, *Manduca sexta* larvae that fed on another transgenic plant silenced in its prosystemin expression consumed more leaf material and became 3 times heavier than those that fed on wild-type plants (Orozco-Cardenas et al., 1993). After wounding, transgenic plants transformed to overexpress the prosystemin gene constitutively produce PI I and II proteins and accumulate more PIs in local and systemic leaves than do wild-type plants (McGurl et al., 1994). Grafting experiments using these overexpressers as root stocks revealed the constitutive production of PIs in wild-type scions (McGurl et al., 1994), indicating the central role of systemin in generating the systemic wound signal in tomato.

More recent grafting experiments between jasmonic acid (JA) biosynthesis mutants (called *spr2* mutants) and wild-type plants, or between systemin signaling mutants (called *spr1* mutants) and wild-type plants, showed that both JA biosynthesis and the presence of systemin are required in the local, wounded leaf to produce the systemic signal and hence to induce PIs systemically. On the other hand, neither JA nor systemin is needed in the systemic, undamaged leaves of tomato plants (Howe, 2004; Schilmiller and Howe, 2005). These findings suggest that systemin acts at or near the site of wounding by amplifying the JA-derived mobile wound signal and are consistent with the previously proposed model that places systemin at the top of the octadecanoid-based signaling pathway.

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upstream of JA (Farmer and Ryan, 1992). Evidence that systemin levels influence the JA levels of a plant was provided by Stenzel et al. (2003), who reported a larger and more rapid rise in JA levels when leaves of prosystemin overexpressing plants were wounded compared to wild-type plants and less JA in plants transformed with an antisense prosystemin construct. Chen et al. (2006) also observed 3-fold higher constitutive JA levels in prosystemin overexpressing plants compared to wild-type plants.

Using the tomato cDNA as a probe, systemin homologs have been found in three other solanaceous species (Constabel et al., 1998), including Solanum nigrum (black nightshade). S. nigrum is a wild relative of potato (Solanum tuberosum) and tomato (Schmidt et al., 2005) and has been established as a model system to study plant-herbivore interactions and their underlying signaling processes. A PI gene called pin2b was found in S. nigrum, which is homologous to and shares 86% sequence similarity with the tomato pill gene. S. nigrum has been shown to locally and systemically induce trypsin-proteinase inhibitor (TPI) activity after wounding (Constabel et al., 1998), as well as to respond systemically by eliciting TPI activity after methyl jasmonate (MeJA) treatment or after attack by the flea beetle Epitrix pubescens (Schmidt et al., 2004)

S. nigrum systemin, which has 83% amino acid identity to the tomato systemin with the respective systemins being 81% identical, induced 10 times less PI I when supplied to excised tomato plants than did tomato systemin itself or any of the other systemin homologs (Constabel et al., 1998). On the other hand, S. nigrum plants supplied with S. nigrum systemin did not accumulate more PIs than control plants supplied with buffer despite a difference in the PI transcript levels between both treatments (Constabel et al., 1998). These findings led us to hypothesize that, in S. nigrum, systemin does not mediate direct systemic defense responses as it does in tomato. To test this hypothesis, we posed the following questions. (1) Which tissues of S. nigrum express systemin constitutively? (2) Does S. nigrum induce systemin after treatment with wounding + M. sexta oral secretions (OS)? (3) Do PIs accumulate differently in wild-type S. nigrum plants than in plants silenced in their prosystemin expression after a wounding + OS elicitation? (4) Does application of systemin induce PI accumulation in S. nigrum? (5) Do herbivores perform differently on wild-type plants than on plants silenced in their prosystemin expression? (6) Do constitutive and induced JA levels differ in S. nigrum wild-type plants from plants silenced in their prosystemin expression?

RESULTS

Spatial and Temporal Prosystemin Transcript Patterns

Prosystemin, of which at least three genes are present in S. nigrum (Supplemental Fig. S2A), was constitutively expressed in all reproductive and vegetative wild-type tissues except for the roots. The sites with the highest expression were the flower buds and the leaves, respectively (Fig. 1, A and B). Interestingly, low prosystemin mRNA levels were detected in black berries and stems (Fig. 1, A and B). After a wounding + OS treatment, the expression of prosystemin decreased rapidly in leaves of wild-type plants and was lowest 30 min after elicitation, whereas, in both lines transformed with an inverted repeat prosystemin construct (IRsys lines), the expression of prosystemin remained very low (Fig. 2, A and B).

TPI Accumulation in Wild-Type and IRsys Plants

To test whether the accumulation of TPI depends on the (pro)systemin level of a plant, the amount of TPIs was quantified in uninduced and induced wild-type and IRsys plants. Although constitutive levels in wild-type and transgenic plants were below the detection limit of the assay, levels increased dramatically after induction (Fig. 3). No significant difference was detected between wild-type and IRsys plants, either in local or in systemic leaves.

Influence of Exogenously Applied Systemin on TPI Levels

As reducing prosystemin mRNA levels in IRsys plants did not reduce TPI accumulation, we tried to
enrich plants with systemin by applying the polypeptide through their cut stems. Applying *S. nigrum* systemin or tomato systemin to wild-type *S. nigrum* plants did not increase TPI levels compared to those of controls (Fig. 4A). However, the application of MeJA was clearly capable of inducing TPIs (Fig. 4A). In tomato plants, the application of tomato systemin significantly increased the level of TPIs compared to control levels, whereas *S. nigrum* systemin did not (Fig. 4B). Using a MeJA dilution series, we demonstrated that *S. nigrum* wild-type plants are able to respond to an exogenously applied elicitor in a dose-dependent manner (Fig. 4A, insert). The patterns resulting from treatment of the IR*Sys* lines did not differ significantly from those of treated wild-type plants (Supplemental Fig. S3).

**Influence of Systemin Levels on Herbivores of Different Feeding Guilds**

To evaluate the influence of systemin on direct defense mechanisms, we compared the performance of herbivores on wild-type plants and IR*Sys* lines. Herbivores from two different feeding guilds, namely, leaf chewers (i.e. caterpillars of the tobacco hornworm *M. sexta* [Sphingidae] and the flea beetle *E. pubescens* [Chrysomelidae]) and phloem sap suckers (i.e. *Myzus persicae nicotianae* [Aphididae]), were chosen. As measures of the leaf quality of the different genotypes, we quantified the mass gain of *M. sexta*, the leaf damage caused by *E. pubescens*, and the population growth of *M. persicae nicotianae*, respectively. The measures of *M. sexta* larval mass and the *E. pubescens* assay were repeated three times. The data shown in Figure 5 are representative for all three experiments. In none of the three herbivore species was a significant difference between wild-type and IR*Sys* lines detected (Fig. 5; all *Ps* > 0.1240).

**Influence of Systemin on JA Levels**

To test whether systemin acts at the top of the octadecanoid pathway upstream of JA, the level of the plant hormone was quantified in the leaves of wild-type plants and IR*Sys* lines treated with wounding + OS. The time series of wild-type plants were characterized by two peaks 30 min and 3 h after elicitation (Fig. 6, insert). The pattern was similar for IR*Sys* lines, with the second peak more prominent than the first. Neither in uninduced nor in induced leaves were significant differences between wild-type and transgenic lines detected (Fig. 6). The wild-type time series as well as the comparison between wild-type plants and IR*Sys* lines were repeated twice with similar results (only one graph is shown).

**DISCUSSION**

The aim of this study was to determine whether systemin’s role in tomato also applies to a solanaceous species that is closely related to tomato. The question was addressed by extending Ryan’s work to *S. nigrum* and testing whether systemin mediates direct systemic defense responses in this species. To compare transgenic *S. nigrum* plants silenced in their prosystemin expression to wild-type plants, we measured TPI and...
JA accumulation after wounding + OS treatment. In addition, we compared the performance of three different herbivore species on wild-type and IRSys plants and observed TPI levels in plants supplied with systemin.

*S. nigrum* harbors at least three prosystemin genes (Supplemental Fig. S2A) that are effectively silenced in both IRSys lines (Fig. 2, A and B). The tissue-specific expression pattern of prosystemin in *S. nigrum* (Fig. 1, A and B) seems to reflect that observed in tomato (McGurl et al., 1992) with high mRNA levels in reproductive and aboveground vegetative tissues. The small amounts of prosystemin mRNA detectable in the black berries (as opposed to the green berries) and roots suggest that systemin is unimportant in these tissues. While the levels of constitutive prosystemin transcript in *S. nigrum* match the levels reported from tomato, the picture changes after induction. In tomato, prosystemin is systemically induced after wounding (McGurl et al., 1992). In contrast, in *S. nigrum* local and systemic transcript levels rapidly decrease after OS elicitation under both glasshouse as well as field conditions (Fig. 2, A and B). To demonstrate that the (pro)systemin levels have an influence on defense responses, PI levels are typically quantified as a response variable in tomato.

The ability of a transgenic tomato line, silenced in its prosystemin expression, to systemically increase PI I and II (McGurl et al., 1992) was almost completely suppressed, indicating a positive correlation between prosystemin expression and PI accumulation. Compared to tomato, the situation is different in *S. nigrum*. Dramatically reducing levels of prosystemin (in both IRSys lines; Fig. 2, A and B) does not reduce TPI accumulation after wounding + OS treatment. In addition, we compared the performance of three different herbivore species on wild-type and IRSys plants and observed TPI levels in plants supplied with systemin.

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We applied tomato systemin to tomato plants, with the expected result: Levels of TPIs significantly increased compared to control levels (Fig. 4B). However, applying *S. nigrum* systemin to tomato did not (Fig. 4B), which was consistent with the findings of Constabel et al. (1998), who pointed out that, in tomato, *S. nigrum* systemin is 10 times less effective than tomato systemin in eliciting PI. Supplying both *S. nigrum* IRSys lines with water or *S. nigrum* systemin did not lead to any significant differences compared to the wild-type plants (Supplemental Fig. S3), supporting the absence of a correlation between systemin and TPIs in *S. nigrum*.

To test the influence of endogenous systemin levels on *S. nigrum*’s resistance to insect attack, the performance of different herbivores on wild-type and IRSys plants was evaluated. Larvae of the tobacco hornworm *M. sexta*, a Solanaceae specialist, gained the same mass when reared on the different genotypes (Fig. 5A). Dramatic differences in weight gain have been reported for tomato plants overexpressing the prosystemin gene compared to wild-type tomato plants (Orozco-Cardenas et al., 1993). Nevertheless, data derived from these overexpressor lines need to be interpreted cautiously as the ectopic expression of prosystemin mRNA driven by an 35S promoter produces systemin in tissues that normally do not express the gene, for example, roots (McGurl et al., 1992). The flea beetle *E. pubescens*, another Solanaceae specialist, has been repeatedly observed to feed on *S. nigrum* at our field site. The mean damage inflicted by this species on the IRSys lines did not differ significantly from that done to wild-type plants (Fig. 5B). Finally, the population growth of *M. persicae nicotianae* did not differ between genotypes (Fig. 5C). Taken together, these results demonstrate that the quality of IRSys and wild-type leaves does not differ for these herbivores.

To test whether the endogenous systemin levels influence a plant’s ability to produce JA, the plant hormone was quantified in both uninduced and OS-elicited wild-type and IRSys plants. Wild-type plants

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**Figure 6.** JA content in leaves of wild-type plants and IRSys lines after wounding + OS treatment. Shown are mean ± se of five individual plants per time point and genotype (single ANOVA followed by LSD post-hoc test per time point: $P > 0.05$). Insert: Shown are mean ± se of five individual wild-type plants per time point.
clearly responded to the treatment (Fig. 6, insert), showing a two-peaked pattern of JA accumulation. The amount of JA did not differ significantly between wild-type and IR Sys plants (Fig. 6), indicating little or no correlation between systemin and JA in S. nigrum. Given that prosystemin transcripts are down-regulated after induction (Fig. 2), systemin and JA may be negatively correlated in S. nigrum, but the low constitutive JA levels in both IR Sys lines argue against this. These data suggest that systemin in S. nigrum does not act at the top of the octadecanoid-based signaling pathway upstream of JA as has been proposed for tomato (Farmer and Ryan, 1992). Current knowledge about systemin based on research in tomato appears not to apply to a closely related member of the same family.

The possibility that systemin might play completely different roles even in closely related species is supported by Boller’s (2005) analysis of systemin sequences, which concluded that systemin appears to be under diversifying selection.

MATERIALS AND METHODS

Plant Growth

The Solanum nigrum inbred line Sn30 (Schmidt et al., 2004) was used as a wild-type control for all experiments. To synchronize germination, seeds of wild-type and transgenic plants were incubated in 5 mL of 1× KOX, supplemented with 30 μL of 0.1 g gibberellic acid (Roth) and 25 μL of 0.5% (v/v) Tween 20 (Merck) at 4°C overnight. Seeds were germinated in Teku pots with a peat-based substrate (Klasmann Tonsubstrat) and transferred to 9-L pots when 9- to 9.5-cm pots containing the same substrate after about 14 d. At all stages the plants were grown in the greenhouse (16 h light, supplemental lighting by Philips Master Sun-T PIA Agro 400 and Sun-T PIA Plus 600 W sodium lights/23°C to 25°C/45% to 55% humidity; 8 h dark/19°C to 23°C/45% to 55% humidity) of the Max Planck Institute for Chemical Ecology (Jena, Germany).

The procedure was the same for tomato (Solanum lycopersicum cv Castlemart), except that the seeds were soaked in water at 4°C overnight.

Plants used in the two field experiments in July and August 2005 (flea beetle herbivory and prosystemin expression after wounding + OS treatment) were planted at the field site in Dornburg (north of Jena, Germany) 24 or 21 d postseeding after being acclimatized to outdoor conditions for 3 to 5 d. The release of transformed plants at the Dornburg field site was conducted in compliance with EU and German regulations (release application nos. 6786–01–0156 [IR Sys line 1] and 6786–01–0165 [IR Sys line 2]) as administered by the Thüringer Landesverwaltungsamt and the Thüringer Landesamt für Lebensmittel sicherheit und Verbraucherschutz.

All experiments were conducted with 4- to 5-week-old plants except those involving the harvesting of reproductive tissues.

IR Sys Lines

Transgenic S. nigrum lines silenced in their prosystemin expression were constructed using the silencing vector pSOL3SY5 (Supplemental Fig. S1), which is based on the pSOLSRCA silencing vector described in detail by Bubner et al. (2006). The original RCA inverted repeat fragments were consecutively replaced by the 399-bp fragments of the S. nigrum prosystemin gene after being PCR amplified using the primer pairs SyS5-32 (5′-CGCGCCATGCTCTGGCATTTTGGGAGG-3′) and SyS6-31 (5′-GGGCCGTCGAGAACATGAGGAGGAGG-3′), respectively, based on the mRNA sequence published by Constabel et al. (1998). Agebacterium tumefaciens-mediated transformation was conducted as described by Krügel et al. (2002), and T1 (transformation generation 1) plants homozygous for the transgene were selected by a hygromycin resistance screen of their progeny. The progeny of homozygous T1 plants was additionally tested to see if it harbored a single copy of the transgene. Southern-blot analysis (Supplemental Fig. S2B) resulted in two independently transformed inverted repeat lines. Seeds of these lines were used for all experiments and will be made freely available to academic investigators for noncommercial research purposes. Line S03-71-13 and line S03-82-3 are referred to as IR Sys line 1 and IR Sys line 2.

Southern-Blot Analysis

Genomic DNA of wild-type and IR Sys plants was isolated from S. nigrum leaves using a modified cetyl trimethyl ammonium bromide method (Rogers et al., 1994) as described by Bubner et al. (2004). The final DNA pellet was rehydrated in 50 μL of 1× Tris/EDTA buffer (10 mM TrisCl, pH 8, 1 mM EDTA, pH 8). Restriction digests were done using BamHI, EcoRV, and EcoRV for the wild-type DNA and EcoRV or XhoI for the DNA of the two IR Sys lines. Plasmids (pSOL3SY5) were linearized using BamHI, XhoI, or Xhol. Size fractionation by 0.8% agarose gel electrophoresis was followed by Southern blotting onto a nylon membrane with a high salt buffer (Brown, 1986). The blots were hybridized with 32P-labeled probes specific for the prosystemin gene (primer pair SYS5-32 [5′-GGCGGCATGGTCTGTCTGCATTTTGGGAGG-3′] and SYS6-31 [5′-GGCGGCATGGTCTGTCTGCATTTTGGGAGG-3′]) or the hygromycin resistance gene hptII.

Plant Treatments

To mimic herbivore feeding, one leaf was wounded with a fabric pattern wheel, causing three rows of punctured wounds on each side of the midrib. The wounds were immediately supplied with OS of Manduca sexta larvae. OS were collected from third- to fourth-instar larvae hatched from eggs (Carolina Biological Supply) and reared on S. nigrum wild-type plants. OS were diluted 1:1 (v/v) with deionized water prior usage. For the prosystemin expression time series, the PI assay, and the JA measurements, a fully expanded leaf of the main axis (normally one leaf at nodes six to eight) was induced as described above. After the respective time points (for the PI assay, after 72 h), the locally treated leaf and in the case of the prosystemin time series the uninduced leaf one node above the treated leaf were harvested, flash frozen in liquid nitrogen, and stored at −80°C until further processing. As the plants grew slower in the field than in the glasshouse, in the field experiment the leaf at the fourth node was treated and harvested as described above. To measure the constitutive prosystemin expression in transgenic plant tissues, the whole plant remained untreated and RNA was extracted from old (third node) leaf blades, petioles, and midribs; young (seventh node) leaf blades, petioles, and midribs; and stems, roots, buds, flowers, and green and black berries.

The PI-inducing effect of S. nigrum and tomato systemin was tested by excising S. nigrum and tomato wild-type plants at the base of the stem according to Pearce et al. (1993) with a scalpel and placing them into tubes containing 500 μL of water, either pure or supplemented with 2.5 pmol systemin (Anaspec; according to protein sequences published by Constabel et al., 1998) or 150 μg of MeJA as a positive control. After the complete solution had been taken up, plants were transferred to water for 72 h and the leaf at the sixth (S. nigrum) or the third node (tomato) was harvested, flash frozen in liquid nitrogen, and stored at −80°C until processing for TPS assay. The experiment was complemented by supplying IR Sys leaves with pure water or water supplemented with 2.5 pmol systemin or 150 μg of MeJA through their cut petioles. To demonstrate that excised S. nigrum leaves are capable of responding to exogenously applied MeJA in a dose-dependent manner, S. nigrum wild-type leaves were supplied with different concentrations of MeJA in 500 μL of water through their cut petioles followed by the procedure described above.

All experiments described above were based on four to six individual plants for each treatment and/or harvest time point. The individual reproductive tissues samples were pooled out of 15 buds, five flowers, three green berries, and two black berries per plant, respectively.

Herbivore Experiments

M. sexta larvae were reared from eggs (obtained from the M. sexta colony at the Max Planck Institute for Chemical Ecology in Jena, Germany) and one neonate was placed on each of the 25 individual plants per genotype (wild type and both IR Sys lines). The caterpillars were weighed after 3, 5, 9, and 11 d.

Naturally occurring adult flea beetles (Epitrix phasianura) were allowed to feed on 45 field-grown plants planted as triplicates of the three genotypes over a period of 10 d. The damage done to the plant was recorded on days 2, 4, and
10. To quantify the damage, each individual leaf was categorized according to the following damage classes: 0 = 0% damage, 1 = 1% to 5% damage, 2 = 6% to 10% damage, 3 = 11% to 25% damage, 4 = 26% to 50% damage, and 5 = 51% to 100% damage. Based on the damage level estimated for each individual leaf, a mean value was calculated for the entire plant. As these mean plant data are noncontinuous percentage values and the damage experienced by wild-type plants was compared singly to the damage done to each IR5s line, thus having two analyses per recording day, Bonferroni-corrected Wilcoxon signed rank tests were performed.

Myzus persicae nicotianae aphids were collected on Nicotiana attenuata plants in our greenhouse and transferred to S. nigrum, where they were allowed to establish a population for about 2 weeks. Single females of this population were used to infest 15 S. nigrum plants of each genotype (wild type and both IR5s lines), and after 10 d the population size on each plant was counted.

**RNA Extraction and Quantitative Real-Time PCR**

Harvested tissues were ground individually in liquid nitrogen and total RNA was isolated following a modified TRI Reagent procedure for polysaccharide- and proteoglycan-rich sources (The Institute for Genomic Research, 2003). Total RNA (100 ng) was reverse transcribed into cDNA using MultiScribe reverse transcriptase (Applied Biosystems) according to the manufacturer’s instructions. Prosystemin mRNA expression was quantified by real-time PCR (ABI PRISM 7700) sequence detection system; Applied Biosystems) using the quantitative PCR core reagent kit (Eurogentec), a specific TaqMan primer pair, and a double fluorescent dye-labeled TaqMan probe specific for the S. nigrum prosystemin gene based on the sequence published by Constabel et al. (1998).

The probe was designed to detect only the endogenous prosystemin gene and not the region selected for the inverted repeat construct. The relative target gene expression of each sample was determined using standard curves. cDNA for these standards was obtained by reverse transcription of S. nigrum RNA extracted from a wounding + OS-treated wild-type plant using SuperScript II RNaseH− reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. With each measurement, TaqMan reactions of four defined cDNA dilutions (20 ng/μL to 0.02 ng/μL) were run using the primers and probe described above. Ct values (the cycle number C at which the PCR product triggers a certain amount of fluorescence [threshold t]) of the four standards were plotted against the respective cDNA concentration and the obtained curve was used to relate the transcript amount of the samples.

**TPI Assay**

Harvested leaves were ground in liquid nitrogen individually and total protein was extracted using 2 mL of protein extraction buffer (longsma et al., 1994) per milligram plant tissue, followed by vortexing and centrifugation as described in Van Dam et al. (2001). Protein content of the samples was determined in technical triplicate using the method of Bradford (1976) with an estimated 1 total protein.

**JA Measurements**

Approximately 300 mg of harvested leaf tissue were homogenized in 1 mL of ethyl acetate spiked with 200 ng mL−1 methanolic [13C2]JA as an internal standard. After centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant was transferred to another tube and the extract was redissolved in 1 mL of ethyl acetate. Following another centrifugation step, the combined supernatants were evaporated and the dried sample was redissolved in 500 μL of 70% (v/v) methanol. After vortexing for 5 min, the sample was centrifuged for 10 min at 13,000 rpm and 15 μL of the supernatant was analyzed using a Variant 120 L triple quadrupole mass spectrometer.

For the HPLC, a Pursuit C8 column (150 mm × 2.0 mm, 3-μm particle size) was used and a gradient of water and methanol, both including 0.05% (v/v) formic acid, was run as the mobile phase with a flow rate of 0.2 mL/min. The mass spectrometer was run in negative electrospray ionization mode with an argon pressure of 0.279972 Pa (≈2.1 mTorr) in the collision cell. The mass spectrometer was set up with a capillary voltage of −3,200 V, a shield voltage of 600 V, and a detector voltage of 1,800 V. The pressure of the drying gas was 131,001 Pa (≈19 psi) at 300°C; that of the nebulizing gas was 379,225 Pa (≈55 psi). The most abundant and characteristic fragment ion was chosen for quantification.

The amount of JA per sample was calculated with the following formula [peak area endogenous JA × 200 ng mL−1] × peak area ISTD−1 and related to 1 mg leaf tissue.

**Statistics**

Data were analyzed by ANOVA followed by an LSD post-hoc test. To ensure homogeneity of variances data were transformed if necessary (square root: Figs. 1A and 2A; LG19 Figs. 5, left, 4A, 7C, and, partly, HR; reciprocal: Figs. 3, right, and 6). In cases in which variances could not be homogenized by transformation, a Welch test followed by a Dunnett T3 test was performed (Figs. 1B, 2B, and 5A). The M. sexta caterpillar mass data were analyzed using repeated-measures ANOVA. All analyses were done using the software package SPSS.

**Supplemental Data**

The following materials are available in the online version of this article.

- Supplemental Figure S1. Silencing vector pSOL3SYS1.
- Supplemental Figure S2. Southern blots of wild type and both IR5s lines.
- Supplemental Figure S3. TPI accumulation in leaves of S. nigrum wild-type plants and both IR5s lines after application of water, S. nigrum systemin (S.n.sys), or MeJA through the cut petioles.

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**LITERATURE CITED**


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