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Plant Biology

Concerted biosynthesis of an insect elicitor of plant volatiles

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ABSTRACT **A variety of agricultural plant species, including corn, respond to insect herbivore damage by releasing large quantities of volatile compounds and, as a result, become highly attractive to parasitic wasps that attack the herbivores. An elicitor of plant volatiles, *N*-(17-hydroxylinolenoyl)-L-glutamine, named volicitin and isolated from beet armyworm caterpillars, is a key component in plant recognition of damage from insect herbivory. Chemical analysis of the oral secretion from beet armyworms that have fed on ^{13}C-labeled corn seedlings established that the fatty acid portion of volicitin is plant derived whereas the 17-hydroxylation reaction and the conjugation with glutamine are carried out by the caterpillar by using glutamine of insect origin. Ironically, these insect-catalyzed chemical modifications to linolenic acid are critical for the biological activity that triggers the release of plant volatiles, which in turn attract natural enemies of the caterpillar.**

Several studies have shown the active role of herbivore-damaged plants in the attraction of insect predators and parasitoids. Volatile plant compounds released in response to insect feeding serve as a chemical signal for herbivore natural enemies (for a review, see refs. 1 and 2). Recent work suggests that this as well as other plant defense responses are triggered by an active component or components associated with the feeding herbivore that allows the plant to differentiate between general wounding and damage caused by chewing insects. In cotton, induced volatiles that are synthesized in response to wounding are released in greater quantities as a result of caterpillar feeding than with mechanical damage alone (3), and, in tobacco, higher concentrations of the defense-signaling molecule jasmonic acid result from herbivore damage by hornworm caterpillars than from mechanical damage designed to mimic herbivory (4). At the transcriptional level, potato mRNAs involved in plant defense accumulate more rapidly with insect-derived elicitor(s) in contact with the damaged leaves than with mechanical damage alone (5).

Thus far, two elicitors of plant volatiles have been identified and reported from chewing insects. Dicke's group has identified a β-glucosidase, present in the regurgitant of *Pieris brassicae* caterpillars that triggers the same emissions of volatiles in cabbage plants as larvae that feed on the plant (6). Because enzyme activity in the regurgitant is retained when caterpillars are fed on β-glucosidase free diet, enzyme activity does not appear to be plant derived. Volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine, identified from the oral secretion of beet armyworm caterpillars, induces corn seedlings to synthesize and release volatile chemical signals (7) that attract parasitic wasps. (ref. 8; for review, see refs. 9 and 10). Volicitin has not been found in plant tissues, although the structure of the molecule suggests that it may interact with the octadecanoid signaling pathway in plants (7). The collection of volatiles from plants fed on by different herbivores suggests that each insect species may produce its own signature molecule(s) that allows plants to differentiate among herbivorous attackers (11, 12). The current study was undertaken to determine the biogenetic origin of volicitin. By feeding beet armyworms corn seedlings labeled with $^{13}CO_2$, we obtained chemical evidence that the caterpillars acquire linolenic acid [an essential fatty acid in the diet of Lepidoptera (13)] from plants and that the insects subsequently hydroxylate and conjugate the fatty acid with glutamine. Thus, the modification by the insect of linolenic acid of plant origin provides a distinct chemical cue that allows a plant to differentiate between herbivore damage and other types of wounding that trigger the octadecanoid defense signaling pathway.

MATERIALS AND METHODS

Plant Growth and Labeling Conditions. Corn seeds, *Zea mays* L., variety LG11 sweet corn, were given 4 days to germinate under a moist paper towel in the dark. To reduce the amount of stored ^{12}C-labeled carbon available for the growing seedling, seeds were trimmed by using a razor blade to remove $\approx$60–70% of the endosperm without cutting into the germinated seedling. Nine excised seedlings were planted in sterilized Metromix 300 potting soil (Scotts-Sierra Horticulture, Marysville, OH). The pot was placed in a 48- $\times$ 16-cm (diameter) glass growth chamber, and synthetic premixed air (Cambridge Isotope Laboratories, Cambridge, MA and Airco, Riverton, NJ), which contained 1,800 μM/liter CO_2 (^{13}C 99%), 20.7% oxygen, and a balance of nitrogen was introduced by flushing the chamber at 500 ml min^{-1} for 10 min and then reducing to a flow of 50 ml min^{-1}. Synthetic air passed up over the potted plants contained within the volatile collection apparatus and out one of eight ports from the glass lid through a plastic tube into a water bubbler. One metal halide and two 400-W high-pressure sodium lamps positioned 10 cm above the chamber provided a 16-h light/8-h dark photoperiod; an air-cooled glass panel directly below the lights insulated the plants from the lamp heat source.

Collection of Plant Volatiles. Corn seedling leaves were damaged with a stainless steel wire, fed through one of the top ports of the growth chamber to scrape the leaves against the glass chamber. Plant volatiles then were collected from 1,200–1,500 h by drawing synthetic ^{13}C-labeled air (100 ml min^{-1}) through Super-Q adsorbent traps (Alltech Associates). Compounds were eluted from the adsorbent filters with 150 μl of dichloromethane; 1-μl aliquots were analyzed by capillary GC on a 50-m $\times$ 0.25-mm (i.d.) fused silica column with a 0.25-μm-thick bonded methyl silicone stationary phase (Quadrex, New Haven, CT). The column was held at 60°C for 5 min and then was increased 5°C per min to 225°C and was held at that temperature for 30 min. Helium was used as a carrier gas at a linear flow velocity of 18 cm sec^{-1}. To determine the amount of ^{13}C incorporated into each compound, samples were analyzed by a Finnigan-MAT ITS40 (ion trap) mass

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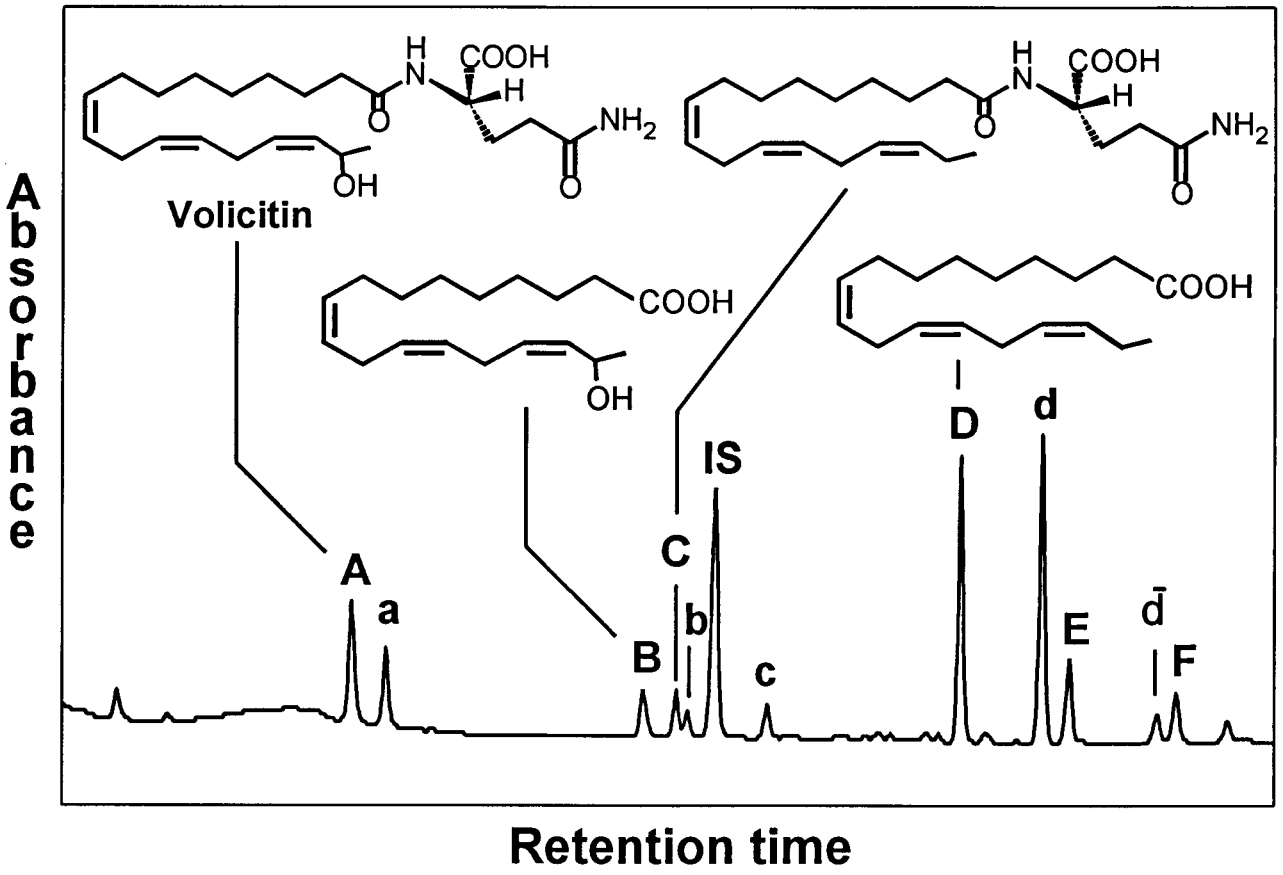

FIG. 1. HPLC profile of volicitin (A) and other fatty acid derivatives detected in the oral secretions of beet armyworms at a wavelength of 200 nm. Compounds and approximate retention times include: (A) *N*-(17-hydroxylinolenoyl)-L-glutamine, 7.5 min; (a) *N*-(17-hydroxylinoleoyl)-L-glutamine, 8.0 min; (B) 17-hydroxy linolenic acid, 11.1 min; (C) *N*-linolenoyl-L-glutamine, 11.9 min; (b) 17-hydroxy linoleic acid, 12 min; (IS) *N*-palmitoleoyl-L-glutamine (internal standard), 12.3 min; (c) *N*-linoleoyl-L-glutamine, 12.6 min; (D) linolenic acid, 15.2 min; (d) linoleic acid, 16.2 min; (E) unknown, 16.6 min; (d̄) oleic acid, 17.1 min; and (F) impurity, 17.8 min.

spectrometer (Finnigan-MAT, San Jose, CA) in the chemical-ionization mode, with isobutane as the reagent gas. Injections were made via a septum-equipped programmable injector held at 40°C for 0.25 min, then programmed at 170°C min^{-1} to 270°C onto a 30-m × 0.25-mm (i.d.) fused silica column with 0.25-μm-thick bonded 5% phenyl methyl silicone (DB-5MS; J & W Scientific, Folsom, CA) held at 40°C for 5 min, then programmed at 5°C min^{-1} to 260°C; He carrier gas linear flow velocity was 19 cm sec^{-1}. Source temperature was adjusted to 120 ± 20°C to optimize the molecular ion abundance. Selected mass ions were quantified via computer software analysis. The fraction of each compound that incorporated ^{13}C was computed on a molecule basis (14).

Plant Chemical Analysis. Lipid and amino acid analysis was based on procedures of Harborne (15). For lipid extraction, frozen plant tissue (0.1 g) was homogenized with chilled isopropanol (5 ml) and was extracted with diethyl ether (5 ml); the supernatant fractions, after centrifugation, were combined and concentrated to dryness. For methanolysis, 2 ml MeOH:HCl (3:1) was added to the extract and was heated for 30 min at 100°C. Hydrolyzed lipids were diluted with H_2O (5 ml), were extracted with CH_2Cl_2 (2 × 4 ml), and were rinsed with saturated $NaHCO_3$ (2 × 4 ml). A concentrated extract was redissolved in a minimum amount of Hex:EtOAc (5:1) and was applied to a 6-ml activated silica gel solid-phase extraction cartridge that was eluted with the same solvent. Fractions containing oleic, linoleic, and linolenic methyl esters initially were identified by comigration with standards on silica gel TLC plates (Merck). Glutamine and glutamic acid were extracted from frozen tissue (0.1 g) with 65% EtOH (5 ml); the supernatant, after centrifugation, was concentrated to dryness and was redissolved in H_2O (400 μl), and the supernatant, after centrifugation, was collected. The aqueous extract was concentrated to dryness and derivatized with 300 μl MeOH:Ac_2O (5:1) by heating for 10 min at 100°C (16). Identification of plant fatty acids, glutamine, and glutamic acid methyl esters were confirmed by comparison with methylated synthetic standards by GC-MS analysis following the same procedures as the analysis of plant volatiles except that samples were injected onto a polar capillary column (OV351, 25-m × 0.25-mm i.d., Quadrex, New Haven, CT), which was held at 60°C for 5 min and then was increased 5°C per min to 230°C and held at that temperature for 30 min.

Caterpillar Regurgitant Collection and Analysis. Beet armyworms were reared on an artificial pinto bean diet following the method of King and Leppla (17). Insects were transferred to feed on corn seedlings at least 48 h before labeling experiments. Regurgitation was induced by holding fourth instar beet armyworm caterpillars with forceps and gently pinching behind the head with a second pair (18). The oral secretion from five larvae was collected, 5 μl of an aqueous *N*-palmitoleoyl-L-glutamine solution (1 $\mu g\ \mu l^{-1}$) was added as an internal standard, and the mixture was centrifuged. The supernatant was fractioned by HPLC [LDC 4100 pump with SM5000 diode array UV detector (LDC Analytical, Rivera Beach, FL)] monitoring wavelength 200 nm on a reversed-phase column (C_{18} ODS-AQ S-5 200 Å, 250 mm long, 4.6-mm i.d., YMC, Kyoto). Components were eluted (1 ml min^{-1}) with a solvent gradient of 40 to 100% CH_3CN, containing 0.8% glacial acetic acid, in H_2O, containing 0.4% glacial acetic acid, over 10 min and then were held at 100% CH_3CN, still

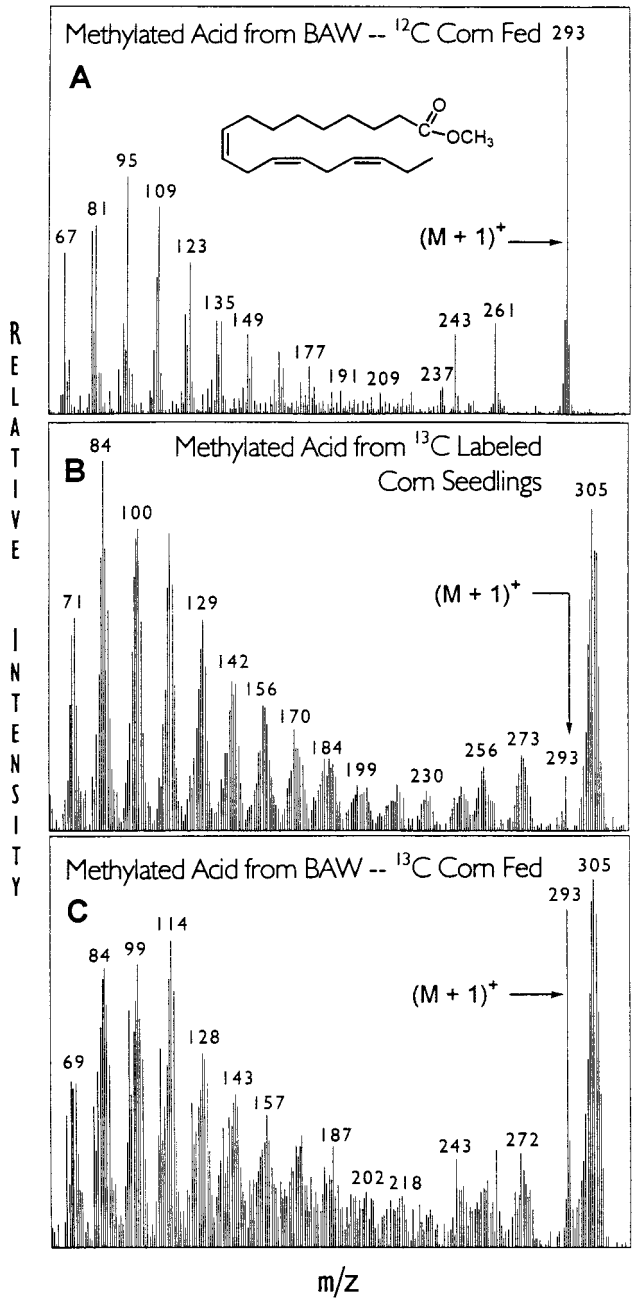

FIG. 2. Chemical ionization mass spectra of methylated linolenic acid from: beet armyworms (BAW) fed on unlabeled corn seedlings for 48 h (*A*), extracted corn seedlings grown for 12 days with $^{13}CO_2$ enrichment (*B*), and beet armyworms fed on unlabeled corn plants and then fed for 6 h on ^{13}C-labeled corn seedlings (C).

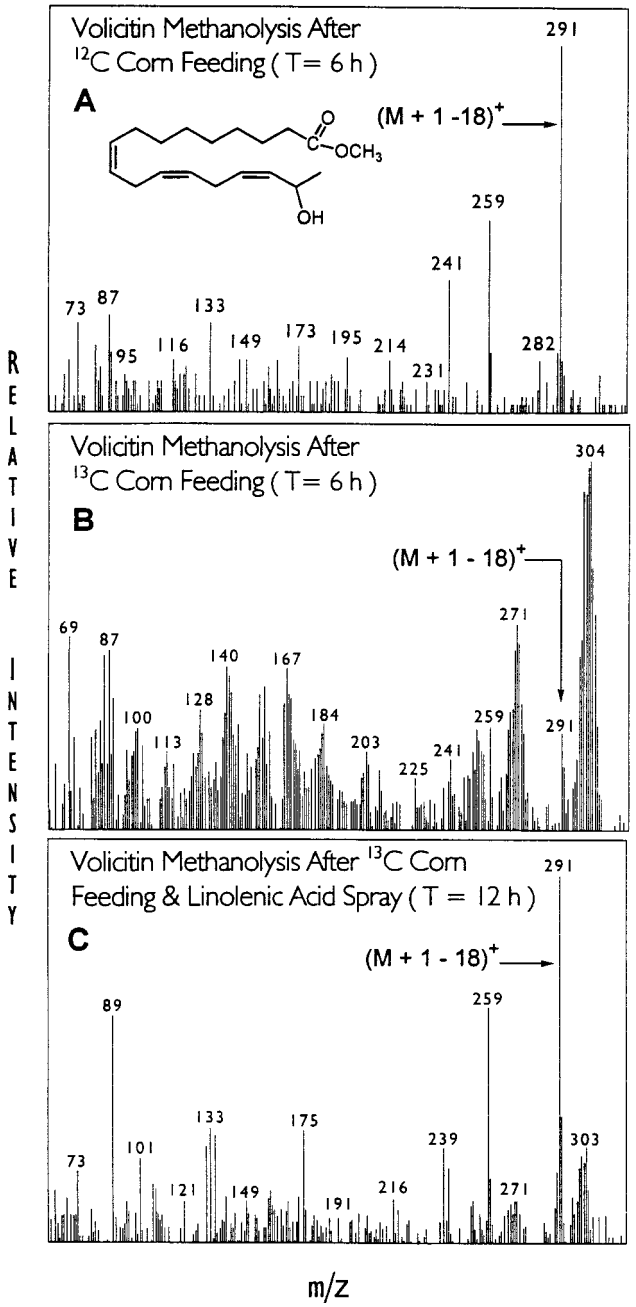

FIG. 3. Chemical ionization mass spectra of methyl 17-hydroxylinolenate prepared by transesterification of volicitin from oral secretion of caterpillars fed 6 h on unlabeled corn seedlings (*A*), 6 h on ^{13}C-labeled corn seedlings (*B*), and 12 h on ^{13}C-labeled seedlings with unlabeled linolenic acid added to the leaves after 6 h (*C*). The 291 ion resulted from loss of water from the molecular ion $(M + 1 - 18)^+$.

containing 0.8% acetic acid, for 10 min. Fractions were methylated with MeOH and Ac_2O as noted above (10).

Unlabeled Linolenic Acid Applications. Linolenic acid (Sigma) in EtOH (1:9) was diluted to a 1% acid solution with H_2O. The solution was applied to labeled corn seedling with an atomize sprayer to cover the corn leaves with a thin mist of unlabeled acid.

RESULTS

To assess rate of synthesis and the source of the chemical components used by beet armyworm (*Spodoptera exigua* Hübner) to assemble volicitin, caterpillars were fed on corn seedlings that were labeled uniformly with ^{13}C. Plants were labeled isotopically by growing seedlings in a closed container into which synthetic premixed air that contained 1,800 μM/liter $^{13}CO_2$ flowed continuously. As a preliminary experiment to estimate the degree of incorporation of ^{13}C into the fatty acids of the plant, leaves were damaged mechanically and the hexenals and hexenols, released as a result of lipoxygenase activity, were collected and analyzed by GC-MS (19). These analyses indicated that linolenic acid in 9-day-old seedlings had incorporated substantial levels of ^{13}C [e.g., 84% enrichment of (*Z*)-3-hexenol]. Seedlings grown for 12 days in an enclosed glass chamber to ensure a high level of ^{13}C labeling were used to feed beet armyworm larvae. Subsequent to the feeding experiment (see below), the remaining portions of the leaves of each plant were extracted and analyzed to determine the

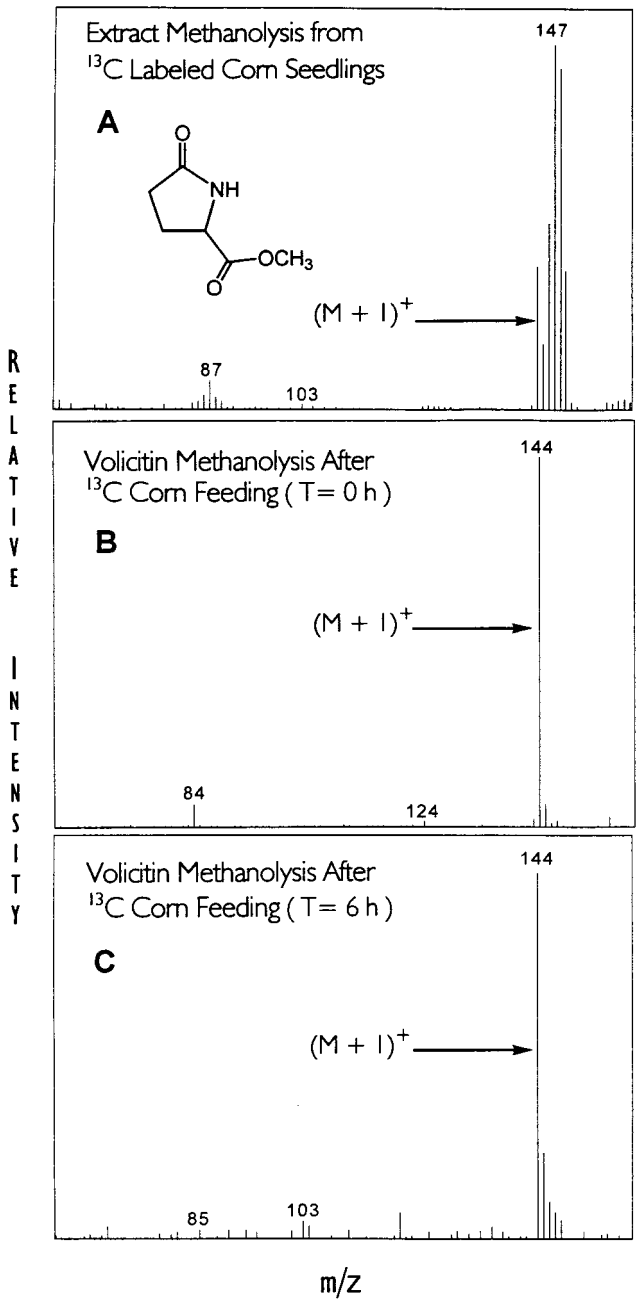

FIG. 4. Chemical ionization mass spectra of methyl pyroglutamate (the product of glutamine methylation) from extracted corn seedlings that were grown for 12 days with $^{13}CO_2$ enrichment (*A*), volicitin from caterpillars that fed on unlabeled corn seedlings for 48 h (*B*), and volicitin from beet armyworms that fed on unlabeled corn plants and then fed 6 h on ^{13}C-labeled corn seedlings (*C*).

percentage of incorporation of the label in linolenic acid and glutamine.

Early fourth instar beet armyworms fed on an artificial diet were transferred to unlabeled corn plants for a minimum of 48 h before being placed on the labeled corn seedlings, to ensure that the ratio of fatty acids in the oral secretions was based on plant constituents and not our rearing diet. Oral secretion was collected by gently squeezing caterpillars after they had fed on labeled seedlings for 6 h, causing them to regurgitate. The oral secretion supernatant from the caterpillars was injected, without further purification, onto a reverse phase HPLC column and was eluted with an acetonitrile-water gradient. Peaks eluting from this column (Fig. 1) were collected, lyophilized, and treated with methanol and acetic anhydride, and the derivatized products were analyzed by GC-MS. Analysis of compounds purified from oral secretions of insects fed on unlabeled and labeled seedlings revealed the consistent presence of nine compounds (Fig. 1). In addition to volicitin, beet armyworm oral secretions contained free 17-hydroxylinolenic acid, *N*-linolenoyl-L-glutamine and free linolenic acid and an analogous series of compounds with a linoleic acid (two double bond) backbone. These compounds have been identified by chemical ionization–MS and fast atom bombardment–MS, and their structures were confirmed by comparisons with synthetic standards (H.T.A., T. H. Jones, G. S. Stenhagen, and J.H.T., unpublished work).

The mass spectral data for the methyl ester of linolenic acid (Fig. 2) as well as the fatty acid portions from the other beet armyworm components demonstrated extensive incorporation of ^{13}C. There are two distinct peak aggregations at m/z 293 and m/z 305 in the spectra of the methyl ester of free linolenic acid from the ^{13}C-labeled corn seedlings and from the caterpillars that have fed on ^{13}C-labeled corn seedlings. The m/z 293 ion $(M+1)^+$ represents linolenic acid molecules devoid of ^{13}C whereas m/z 311 $(M+19)^+$ corresponds to linolenic acid in which all carbon is ^{13}C-labeled. The fraction of the fatty acid portion of each compound that incorporated ^{13}C on a molecular basis with 6 h of feeding on the labeled corn seedlings is as follows: *N*-(17-hydroxylinolenoyl)-L-glutamine, 63%; *N*-(17-hydroxylinoleoyl)-:L-glutamine, 52%; 17-hydroxylinolenic acid, 61%; *N*-linolenoyl-L-glutamine, 83%; *N*-linoleoyl-L-glutamine, 51%; linolenic acid, 80%; and linoleic acid, 57%. In fact, within 6 h, the acids in beet armyworm oral secretions contained a level of ^{13}C-labeling comparable to what is found in the labeled seedlings. For the fatty acids analyzed from the caterpillars, as well as the labeled corn seedlings that they fed on, the pattern of ^{13}C incorporation followed a gaussian distribution with m/z 305 representing slightly more than half of the carbon atoms containing the ^{13}C label. This indicates that the fatty acid portion of volicitin and the other conjugated compounds in the insect oral secretion are obtained directly from the plant.

To determine whether the beet armyworm hydroxylates the fatty acid portion of volicitin or whether the entire 17-hydroxylinolenic acid molecule is plant-derived, a pulse–chase experiment was conducted. Caterpillars that had fed on ^{13}C-labeled corn for 6 h so that the linolenic acid moiety of volicitin was ^{13}C-labeled (Fig. 3*B*) were moved to ^{13}C-labeled seedlings that had been sprayed with unlabeled linolenic acid and were allowed to feed for an additional 6 h. The marked decrease of ^{13}C in the methyl 17-hydroxylinolenoate derived from volicitin (Fig. 3*C*) and free linolenic acid, both collected from the beet armyworm, indicates that hydroxylation of the relatively large amount of free unlabeled linolenic acid sprayed onto the plant leaves is done by the beet armyworm. The linolenic and linoleic acid components from caterpillars that had fed on unsprayed ^{13}C-labeled seedlings for the same total time of 12 h maintained high ^{13}C incorporation levels. To ensure that the decrease in ^{13}C incorporation with application of unlabeled linolenic acid was a specific response for linolenic acid derivatives, linoleic acid was collected from caterpillars that had fed on sprayed seedlings and was found to sustain the high level of ^{13}C labeling. The 17-hydroxylinolenic acid methyl ester and the 17-hydroxylinoleic acid methyl ester were not detected in derivatized plant extracts, although these compounds were identified when plant tissue was spiked with the corresponding synthetic acid before extraction and methylation.

In contrast to the fatty acid portion of volicitin, which rapidly incorporated ^{13}C and showed a similar mass spectral labeling pattern as the linolenic acid from the plant (Fig. 2*B*), the glutamine incorporated little ^{13}C relative to the glutamine from the plant (Fig. 4), indicating that the plant was not catalyzing the coupling of glutamine to the fatty acids. It also appears that labeled glutamine from the plant ingested by the

insect was diluted with an unlabeled insect source of glutamine before coupling to form the volicitin molecule. This could be caused by either a large glutamine pool present in the caterpillar's oral secretions or a physically separate glutamine source. Because the plants are ^{13}C-labeled from the time that photosynthesis begins, the possibility that unlabeled glutamine is derived from an isolated source separate from current photosynthate and apart from the major pool of plant glutamine is unlikely.

These biochemical data demonstrate that the plant supplies linolenic acid, which is required for growth and development of beet armyworms, and also provides this fatty acyl chain for the synthesis of volicitin, the modified elicitor of plant volatiles that is central to signaling between plants and natural enemies of the caterpillars that attack them. It is not clear why the caterpillar (or gut symbionts in the caterpillar) adds L-glutamine and the hydroxy group to linolenic acid or whether volicitin plays a role in the metabolism of the herbivore. It is also not clear how volicitin interacts on a biochemical level to induce synthesis of plant volatiles; however, we do know that volicitin provides a rapid, clear, and reliable signal for initiation of synthesis and release of volatile compounds. Thus the plant and the herbivore are inexorably linked through a signaling molecule of dual origin and effect.

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