an agar plate solidified with 1% agar. These leaves were covered by a black plate with open slits approximately 1 mm in width arranged in parallel and irradiated through these slits with strong cool white light (~30 W m^{-2}) for 1.5 hours using a metal halide lamp. The area of wild-type leaves irradiated through the slit became pale green, while the covered area remained dark green (Fig. 1A). The light-induced change in the green color is a consequence of the movement of chloroplasts to the side walls of cells. Thus, the color change will not be observed in mutant leaves lacking the avoidance response. Mutants *cav1-1* to *cav1-3* from EMS-treated seeds and *cav1-4* from T-DNA–tagged lines were screened by this method.

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10. The NPL1 knockout line was isolated from 13,440 T-DNA–tagged lines (WS ecotype). One super pool of genome DNA, prepared by Kazusa DNA Research Institute, included 384 lines, and 35 superpools were used for the first screening. Screening was done by the PCR method, using *NPL1*- and T-DNA–specific primers.
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14. T. Sakai, K. Okada, unpublished data.
15. Preparation of the seedlings and RNA gel blotting for the analysis of the *NPL1* gene expression was done as described (*22*). For the analysis using various light qualities, white light was provided by a white fluorescent lamp (model FL20SSW/18; National, Tokyo) at the fluence rate of 30 μmol m^{-2} s^{-1}. Blue and red light were provided by a light-emitting diode (LED), blue light lamps, and red light lamps (LED-mB; LED-mR; Eyela, Tokyo) with a maximum wavelength of 470 nm (30 nm half band-width) and of 660 nm (20 nm half band-width) at the fluence rate of 10 μmol m^{-2} s^{-1}, respectively. Quantitative RT-PCR was done by using ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) with TaqMan EZ RT-PCR Kit (PE Applied Biosystems). RNA samples (100 ng and 1 ng) treated by deoxyribonuclease I were used for the quantitative RT-PCR of *NPL1* mRNA and 18*S* rRNA, respectively. Nucleotide sequences of PCR primers for the *NPL1* gene were 5′-GAGACGCAATTAGAGATCAGAGG-3′ and 5′-CCGATGAAGTATTGAAGCTCTC-3′. Sequence of the probe to quantify the RT-PCR product was a 5′-CCACTTGCAACCTATGCGTGATCAGAAG-3′. This probe had a FAM (6-carbosyfluorescein) as a 5′-reporter dye and a TAMRA (6-carbosy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine) as a 3′-quencher dye. RT-PCR primer and the probe for 18*S* rRNA were used of TaqMan Ribosomal RNA Control Reagents (PE Applied Biosystems).
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23. We are grateful to W. R. Briggs and J. M. Christie of Carnegie Institution of Washington (Stanford, CA) and J. A. Banks of Purdue University (West Lafayette, IN) for critical reading of the manuscript. This work was partly supported by PRESTO, Japan Science and Technology Corporation, Japan, to T.K. and by grants from PROBRAIN (Program for Promotion of Basic Research Activities for Innovative Biosciences), Mitsubishi Foundation, Grant-in-Aid for International Scientific Research (Joint Research, no. 10044214) and for Scientific Research (B, no. 09440270) from the Ministry of Education, Sports, Science and Technology (MEXT) of Japan to M.W. Also supported by a grant from the program Grants-in-Aid for Scientific Research on Priority Areas (no. 10182101); a fund from the Joint Studies Program for Advanced Studies from MEXT, the Human Frontier Science Program, the Mitsubishi Foundation to K. Okada; and by funds from the Japan Society for the Promotion of Science to T.S. as a research fellow.

29 September 2000; accepted 18 January 2001

Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature

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Herbivore attack is known to increase the emission of volatiles, which attract predators to herbivore-damaged plants in the laboratory and agricultural systems. We quantified volatile emissions from *Nicotiana attenuata* plants growing in natural populations during attack by three species of leaf-feeding herbivores and mimicked the release of five commonly emitted volatiles individually. Three compounds (*cis*-3-hexen-1-ol, linalool, and *cis*-α-bergamotene) increased egg predation rates by a generalist predator; linalool and the complete blend decreased lepidopteran oviposition rates. As a consequence, a plant could reduce the number of herbivores by more than 90% by releasing volatiles. These results confirm that indirect defenses can operate in nature.

Plants defend themselves against herbivores with chemical and physical defenses that directly influence herbivore performance and indirectly through traits that attract the natural enemies of herbivores (*1*–*3*). One such indirect defense, the release of volatile organic compounds (VOCs) specifically after herbivory, is known to attract parasitoids and predators to actively feeding larvae in the laboratory (*4*, *5*), and evidence from agricultural systems suggests a role for herbivore-induced VOCs in increasing predation pressure (*6*–*8*). However, conclusive evidence has been lacking, and it is not even known whether plants growing in natural populations increase VOC emissions after herbivore attack. VOCs might be able to function as indirect defenses only in simplified agroecosystems, in which a single natural enemy species of a herbivore can act as an important biocontrol agent on an agricultural plant (*9*). In contrast, in natural systems, herbivore mortality is more commonly mediated by a suite of generalist enemies (*10*). Moreover, both the qualitative and quantitative characteristics of herbivore-induced plumes of VOCs are known to vary among plant genotypes (*11*–*13*); the genetic variation commonly found in natural populations may undermine the reliability of VOCs as a signal for natural enemies because prior exposure is often needed to associate plant VOCs with the occurrence of a feeding herbivore (*5*, *14*, *15*). Herbivore-induced plant VOCs may also influence herbivore host-location behavior, potentially increasing herbivore attack on plants releasing VOCs (*1*, *2*).

To evaluate the role of herbivore-induced VOCs in nature, we characterized the VOCs released from *Nicotiana attenuata* Torr. ex Wats (Solanaceae) plants growing in a native population (*16*–*18*) in the Great Basin desert of southwest Utah, which were under continuous attack by three numerically dominant folivores: the caterpillars of *Manduca quinquemaculata* (Lepidoptera, Sphingidae), the leaf bug *Dicyphus minimus* (Heteroptera, Miridae), and the flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysomelidae) (*19*). We used an open-flow trapping design (*13*) to collect VOCs individually from 32 plants growing in a natural population that each had one leaf attacked by one of the three herbivore species or remained undamaged (*13*, *20*) (Fig. 1, A and B). All plants were growing in a 150-m^2 portion of the population (*18*) and were sampled simultaneously for 7 hours. Gas chromatography–mass spectrometry (GC-MS) analysis (*21*) of the trapped VOCs revealed that all three herbivore species elicited increases in the same suite of VOCs, although the odor profiles were not identical (Fig. 1B). The pattern and amount of herbivore-induced VOCs trapped from *N. attenuata* growing in the field were very similar to those found in laboratory studies with plants attacked by *Manduca sexta* larvae (*13*).

The emitted VOCs common to all three herbivores are derived from three biosynthetic pathways. Green leaf volatiles (*cis*-3-hexene-1-ol, *cis*-3-hexenyl acetate, and *cis*-3-hexenyl butyrate; 1, 3, and 6, respectively, in Fig. 1) derived from the octadecanoid path-

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way are known to be emitted rapidly after damage (*11*, *13*) but are not specific to plants attacked by herbivores (*2*). The terpenoids (*trans*-β-ocimene, *cis*-α-bergamotene, and *trans*-β-farnesene; 2, 8, and 9, respectively, in Fig. 1) are emitted more slowly, typically 24 hours after attack (*11*, *13*). Only 5 of the 24 attacked plants emitted linalool (4 in Fig. 1), which is consistent with previous findings that only some *N. attenuata* genotypes are linalool emitters (*13*). All attacked plants showed significantly elevated emissions of *cis*-α-bergamotene, which is known to be elicited in *N. attenuata* when a suite of seven to eight fatty acid–amino acid conjugates that occur in the oral secretions of both *M. sexta* and *M. quinquemaculata* larvae are introduced to leaf wounds (*22*) as well as when plants are treated with methyl jasmonate (MeJA) (*13*). Finally, the emission of the shikimate-derived methyl salicylate (MeSA; 7 in Fig. 1) was significantly elevated in the headspace of all attacked plants. Because all of these compounds have been identified in the headspace of other herbivore-infested plants (*2*, *5*, *23*, *24*), they may function as universal signs of herbivore damage, and we hypothesized that increasing the emission of single compounds in the context of the plants' natural background emission should attract natural enemies in nature.

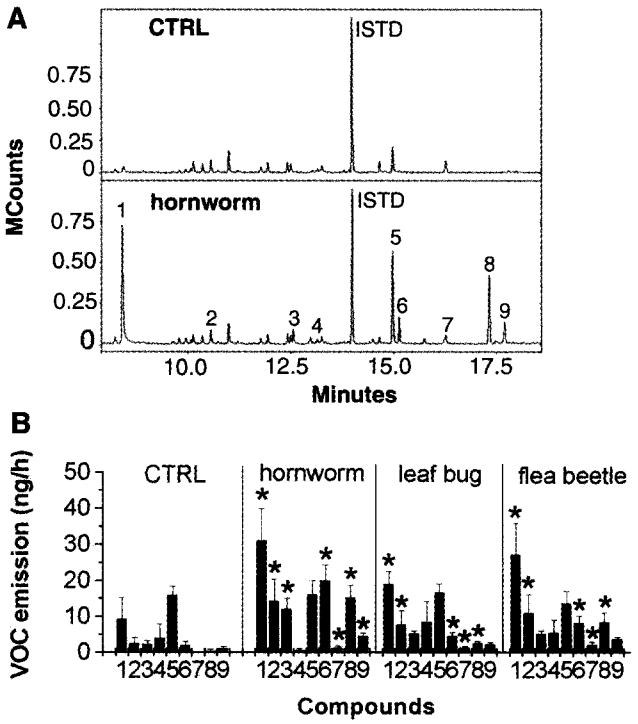

Fig. 1. VOC release in response to herbivory in nature. (**A**) Representative total ion chromatograms of the headspace of an undamaged leaf from an undamaged plant (CTRL) in comparison with a leaf damaged by a *M. quinquemaculata* larva (hornworm). (**B**) Comparison of the mean (±SEM) emissions of VOCs from undamaged plants (CTRL) and plants damaged by *M. quinquemaculata* larvae (hornworm), *D. minimus* (leaf bug), and *E. hirtipennis* (flea beetle) (with eight replicate plants per treatment in the same native population). Because herbivore-induced VOCs from *N. attenuata* are emitted with different kinetics depending on the compound (*13*), data from the 7-hour trapping, 24 hours after herbivore infestation, are presented. Each VOC trap was spiked with 300 ng of tetraline as an internal standard (ISTD), eluted with 750 μl of dichloromethane, and analyzed by GC-MS (*21*). The labels in both (A) and (B) represent the following: 1, *cis*-3-hexen-1-ol; 2, *trans*-β-ocimene; 3, *cis*-3-hexenyl acetate; 4, linalool; 5, terpineol; 6, *cis*-3-hexenyl butyrate; 7, methyl salicylate; 8, *cis*-α-bergamotene; and 9, *trans*-β-farnesene. Asterisks designate compounds whose emission was significantly [$P < 0.05$; Bonferroni-corrected Fisher's protected least significant difference (LSD) test of an analysis of variance (ANOVA)] elevated in comparison with undamaged control plants.

To test this hypothesis, we mimicked the herbivore-induced emission of individual compounds from each biosynthetic class. To mimic the volatile release, we applied 200 μg of either MeJA, MeSA, *cis*-3-hexene-1-ol, *trans*-β-ocimene, *racemic* linalool, or *cis*-α-bergamotene in 20 μl of lanolin paste to the stems of flowering plants in a natural population (*18*). Controls were treated with 20 μl of pure lanolin. To determine the fidelity of the mimicry, we trapped the VOCs from whole plants and found the emission to be very similar to those of herbivore-infested or MeJA-treated plants (Fig. 2A: data shown for control, MeJA-, and *cis*-α-bergamotene–treated plants). Application of individual compounds (with the exception of MeJA, which elicited a majority of herbivore-induced VOCs) resulted in increased emissions of only the applied compound, which was released in quantities within the range of emissions observed in herbivore-infested plants (Fig. 2A) (*25*).

We used *M. sexta* eggs to measure predation rates to avoid the confounding influence of direct defenses elicited by herbivore feeding. A single hornworm larva can completely defoliate 1 to 10 reproductively mature *N. attenuata* plants in the course of its development, and hornworm larvae have been responsible for most of the leaf area lost to insect herbivores in six *N. attenuata* populations monitored in southwest Utah over the past 2 years. *Manduca* eggs are typically laid singly on plants, but clusters of four to seven eggs on a single leaf are occasionally found. To measure predation rates, we fixed five *M. sexta* eggs to the underside of both the second and third stem leaves (*26*) of 105 unattacked *N. attenuata* plants, using a neutral α-cellulose glue that was known not to effect changes in VOC emissions (*27*). Lanolin paste with and without the individual VOCs was placed on the stem between the leaves.

Plants were of the same size (30 to 40 cm), unattacked, and 3 to 5 m apart in a linear 500-m transect across a population of more than 100,000 plants. Treatments were applied so that no two plants of the same treatment were within 21 to 35 m from each other. This distance was sufficient to prevent the plumes from similarly treated plants from interacting and provided a conservative measure of the ecological function of VOC emission. In another experiment in the same population of plants, in which distances between similarly treated plants were only 3 to 5 m of each other, predation rates were 13-fold higher (Fig. 2B; data for MeJA and control treatments shown), probably because of the lack of independence of replicates within a treatment.

During the experiment, only one predator (*Geocoris pallens*; Heteroptera, Geocoridae) was observed feeding on the eggs and hatching larvae. Attacked eggs are emptied and hence easy to distinguish. Moreover, this predator was repeatedly observed preying on leaf bugs and flea beetles. During the previous field season, *G. pallens* was responsible for 95% of the *M. quinquemaculata* and *M. sexta* larvae mortality (*26*); no *Manduca* eggs on *N. attenuata* were parasitized. Survival of eggs and neonatant larvae was monitored for 48 hours. After 24 hours, the mortality was already significantly higher on plants treated with MeJA (36 ± 5.4%), *cis*-3-hexene-1-ol (33.8 ± 6.7%), linalool (37.5 ± 5.3%), and *cis*-α-bergamotene (33.3 ± 5.1%), whereas mortality on plants treated with MeSA (21.2 ± 5.8%) and *trans*-β-ocimene (28.7 ± 5.8%) did not differ significantly from mortality on untreated control plants (16.7 ± 4.4%; Fig. 2B).

To determine if the herbivore-induced release of VOCs influenced the oviposition behavior of native adult *Manduca* moths, we established a new transect with unattacked plants across the same populations with the following treatments (*28*): one to four foraging *M. quinquemaculata* first to third instar larvae per plant, MeJA to elicit a majority of the herbivore-induced VOCs, one green leaf VOC (*cis*-3-hexenyl butyrate) and one terpenoid (linalool), and a control lanolin treatment. At the time of the experiment, *M. quinquemaculata* adults

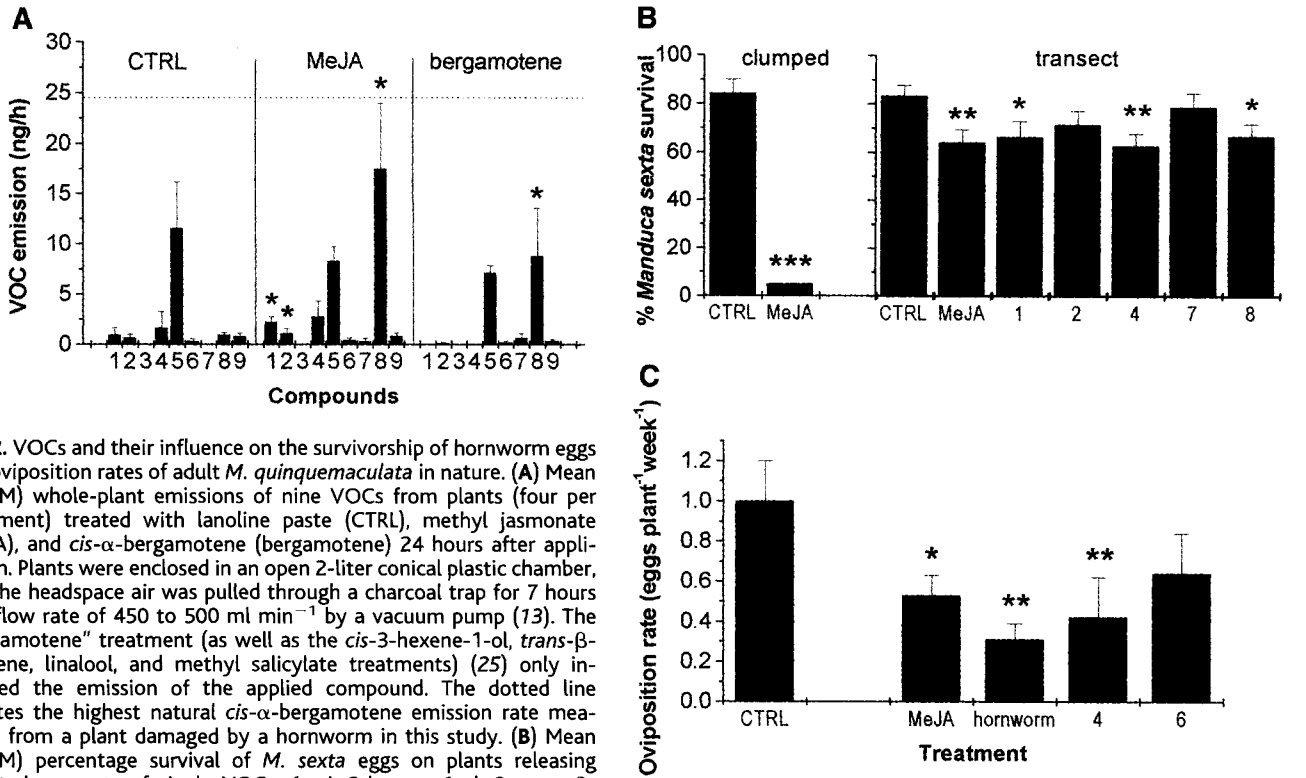

Fig. 2. VOCs and their influence on the survivorship of hornworm eggs and oviposition rates of adult *M. quinquemaculata* in nature. (**A**) Mean (±SEM) whole-plant emissions of nine VOCs from plants (four per treatment) treated with lanoline paste (CTRL), methyl jasmonate (MeJA), and *cis*-α-bergamotene (bergamotene) 24 hours after application. Plants were enclosed in an open 2-liter conical plastic chamber, and the headspace air was pulled through a charcoal trap for 7 hours at a flow rate of 450 to 500 ml min^{-1} by a vacuum pump (*13*). The "bergamotene" treatment (as well as the *cis*-3-hexene-1-ol, *trans*-β-ocimene, linalool, and methyl salicylate treatments) (*25*) only increased the emission of the applied compound. The dotted line denotes the highest natural *cis*-α-bergamotene emission rate measured from a plant damaged by a hornworm in this study. (**B**) Mean (±SEM) percentage survival of *M. sexta* eggs on plants releasing elevated amounts of single VOCs: 1, *cis*-3-hexene-1-ol; 2, *trans*-β-ocimene; 4, linalool; 7, methyl salicylate; and 8, *cis*-α-bergamotene. In the "clumped" experimental design, plants treated with the same VOC were within 3 to 5 m of each other, whereas in the "transect" experimental design, similarly treated plants were within 21 to 35 m of each other. (**C**) Mean (±SEM) *M. quinquemaculata* oviposition rates on MeJA-treated plants, plants under previous attack by hornworms, and plants treated with *cis*-3-hexenyl butyrate (6) and linalool (4). Asterisks represent significant differences from control (CTRL) plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as determined by Fisher's protected LSD test of an ANOVA.

were ovipositing, and the number of eggs deposited on each plant was counted and removed every second day for 14 days. Lanolin treatments were refreshed every 48 hours to maintain VOC emissions. Over a 1-week period, moths laid fewer eggs per plant on plants under attack by caterpillars (0.31 ± 0.08), on plants treated with MeJA (0.53 ± 0.1), and on plants treated with and releasing linalool (0.42 ± 0.2). Plants treated with *cis*-3-hexenyl butyrate (0.64 ± 0.2) received the same number of eggs as control plants (1.0 ± 0.2) (Fig. 2C).

By releasing VOCs after herbivore attack, a plant can profoundly influence both oviposition and predation rates in nature and thereby influence both "bottom-up" as well as "top-down" control over its herbivore populations. The emission of linalool alone caused a 2.4-fold reduction in oviposition rate. Daily predation rates on plants releasing VOCs, extrapolated to 1 week, were 4.9 to 7.5 times higher than those observed on control plants; hence, the top-down effects in this experiment were more strongly influenced by VOC emissions. The multiplicative effect, calculated from both the bottom-up and the top-down components of this indirect defense (*25*) could reduce the numbers of the plant's most significant insect folivore, *M. quinquemaculata*, by 91.7 (MeJA treatment) and 94.5% (linalool treatment). Herbivores as well as predators appear to use the same volatile signals, suggesting that plants are under strong selection to release them.

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18. *N. attenuata* is a postfire annual plant that can dominate the pioneer community 1 to 3 years after a fire destroys the dominant vegetation and stimulates germination from a long-lived seed bank. Experiments were performed during the 2000 growing season (April to August) with a large population growing on a 2.72-km^2 area (T43SR17W section 32) west of St. George, Utah, which burned for 7 days after a lightning strike on 17 June 1999 (fire number W246).
19. Quantitative census of the herbivorous arthropod community residing on 250 *N. attenuata* plants at each of two burns during the 2000 field season revealed the following percentage representations: *D. minimus* (52.9%), *E. hirtipennis* (18.3%), *Geocoris spp.* (9.6%), *M. quiquemaculata* (9.2%), *M. sexta* (3.3%), and others (5.8%). Although both *Manduca* species co-occurred on *N. attenuata* plants, *M. quinquemaculata* was more abundant earlier in the season.
20. Previous laboratory experiments with *N. attenuata* revealed that whole-plant VOC emissions were quantitatively related to the amount of herbivore-specific leaf damage. Hence, we infested plants with the following numbers of the three herbivore species to release comparable amounts of VOCs: eight adult *D. minimus*, five adult *E. hirtipennis*, and one third instar *M. quinquemaculata* larva (*29*). To restrict insects to a single leaf and to trap volatiles from this leaf, we enclosed leaf and insects in 400-ml polystyrene chambers fitted with holes at both ends. Air was pulled through the chamber at 450 to 500 ml min^{-1} (measured by a mass flow meter; Aalborg Instruments, Orangeburg, NY) and subsequently through a charcoal air sampling trap (ORBO™-32; SUPELCO, State College, PA) by a portable 12-V DC vacuum pump (Gast Mfg, Benton Harbor, MI). Measurements inside and outside the chambers revealed no temperature differences throughout the 2 days of 14-hour sampling.
21. Analysis was performed at the field sites with a Shimadzu (Model 5000) quadrupole GC-MS programmed as follows: injector 225°C, initial column

temperature held at 45°C for 6 min, increased at 10°C min^{-1} to 130°C, increased at 5°C min^{-1} to 180°C, increased at 20°C min^{-1} to 230°C, held for 5 min, increased at 20°C min^{-1} to 250°C, and held for 5 min. Helium carrier gas flow was set to 1 ml min^{-1} with an electronic pressure control unit. Spectra were collected at −70 eV, and compounds were identified by comparison of retention times and mass spectra with those of authentic standards.

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25. A comparison of natural VOC emission with emissions from plants treated with individual VOCs as well as a detailed summary of observed and calculated values of top-down and bottom-up effects are available as supplementary data at *Science* Online at www.sciencemag.org/cgi/content/full/291/5511/2141/DC1.
26. We chose to attach *M. sexta* eggs on the underside of second and third stem leaves (measured from the bottom of the plant) to measure predation rates because an extensive study during the 1999 field season of mortality factors of both *Manduca* species revealed that predation rates depended on leaf position. The probability of predation for eggs and first to third instar hornworms on rosette leaves was 2.6 times higher than that on the second to fourth stem leaves and 11.8 times higher than that on the fifth or younger stem leaves (*29*). We followed the fate of 174 naturally oviposited hornworm eggs and could ascribe about 95% of the mortality to *G. pallens* predation. The decreasing predation rates of eggs and larvae feeding at higher leaf positions were likely due to the ground-dwelling behavior of *G. pallens*. We monitored the mortality of 559 naturally oviposited eggs in the 1999 field season, of which 71.75% were laid on first to fourth leaf position. About half of the mortality occurred at the egg stage (50.5%), 31.5% at first instar larvae, and 17.4% at second instar larvae (*29*).
27. Quantities of VOCs trapped for 7 hours from whole plants 24 hours after treatment with the α-cellulose glue used to fix eggs to leaves in the field experiment did not significantly increase any VOC emissions as compared with water-treated control plants (all *P* for compounds 1 to 7 >0.88). Laboratory experiments also demonstrated that naturally oviposited *M. sexta* eggs did not elicit a detectable plant VOC release in comparison with control plants without eggs (*29*).
28. We used 40- to 50-cm-tall flowering plants, growing 3 to 5 m apart in a 400-m linear transect across the population.
29. A. Kessler, unpublished results.
30. Supported by Max-Planck-Gesellschaft. We thank P. Feeny, J. Gershenzon, M. Hilker, J. McNeil, F. Roces, A. Roda, E. Wheeler, and three anonymous reviewers for helpful comments; B. Krock and H. Thomas for purifying the *cis*-α-bergamotene; R. Baumann for assistance with species determinations; and Brigham Young University for use of Lytle Preserve as a field station.

31 October 2000; accepted 6 February 2001

A Sperm Cytoskeletal Protein That Signals Oocyte Meiotic Maturation and Ovulation

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Caenorhabditis elegans **oocytes, like those of most animals, arrest during meiotic prophase. Sperm promote the resumption of meiosis (maturation) and contraction of smooth muscle–like gonadal sheath cells, which are required for ovulation. We show that the major sperm cytoskeletal protein (MSP) is a bipartite signal for oocyte maturation and sheath contraction. MSP also functions in sperm locomotion, playing a role analogous to actin. Thus, during evolution, MSP has acquired extracellular signaling and intracellular cytoskeletal functions for reproduction. Proteins with MSP-like domains are found in plants, fungi, and other animals, suggesting that related signaling functions may exist in other phyla.**

In sexually reproducing metazoans, oocyte meiotic cell cycle progression is coordinated with ovulation and fertilization to ensure fusion of haploid gamete nuclei. In many animals, sperm trigger the resumption of meiosis in arrested oocytes, but the underlying mechanisms are not clear. During *C. elegans* reproduction, sperm promote oocyte meiotic maturation (M-phase entry) and gonadal sheath cell contraction, which act in concert to facilitate ovulation (*1*). Fertilization then occurs as ovulating oocytes enter a sperm storage compartment called the spermatheca (Fig. 1A). *Caenorhabditis elegans* sperm are separated from oocytes and sheath cells by a valve-like constriction of the distal spermatheca. Therefore, we reasoned that sperm likely secrete factors that promote both oocyte maturation and sheath contraction. To identify the sperm signals, we developed an in vivo bioassay by microinjecting sperm-conditioned medium (SCM) (*2*) into the uterus of *fog-2(q71)* females (*3*) (Fig. 1A). The oocyte maturation and sheath contraction rates are very low in these mutants (*1*), which lack sperm due to a defect in germline sex determination (*3*). Microinjection of SCM into *fog-2* females causes robust increases in the oocyte maturation and sheath cell contraction rates, as visualized by time-lapse video microscopy [Web movies (*4*)]. Sheath cells also respond with an increased contraction intensity, as measured by their lateral displacement. No activity is observed after the microinjection of bacterial extracts, female extracts, 1-methyladenine, serotonin, oxytocin, or M9 buffer.

The bioactive factors present in SCM are heat-resistant (100°C, 20 min) and sensitive to proteinase K digestion, suggesting that they are proteinaceous. Comparison of SCM to sperm lysates by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) indicates that SCM is highly enriched with a single protein (Fig. 1B). The bioactive factors were purified with reversed-phase high-performance liquid chromatography (HPLC) using C_4 and C_{18} columns (*5*). Collected fractions were dialyzed in M9 buffer and assayed individually. Single peaks of maturation- and contraction-inducing activity elute from both columns (Fig. 1C). The identical separation characteristics of both activities on two columns with gradient elution indicates that both activities are likely contained in the same protein or protein complex. MALDI-TOF mass spectrometry shows that a polypeptide of 14,121 ± 1 dalton is present in the active fractions (Fig. 1D). Tryptic peptide mapping and sequencing representative fragments with post source decay mass spectrometry identify the bioactive polypeptide as the major sperm protein (MSP) (*6*). *Caenorhabditis elegans* MSP variants differing by one to four amino acids are encoded by a multigene family of approximately 40 genes (*7*). Closer analysis of the mass spectra reveals that several isoforms with similar molecular weights are present. Two of the major peaks match the calculated molecular weights of MSP-3 and MSP-142 (Fig. 1D). Nanomolar concentrations of SCM-purified MSP cause dramatic increases in the oocyte maturation and sheath cell contraction rates when microinjected into the uterus of *fog-2* females (Fig. 2). MSP, purified from sperm lysed with glass beads, produces identical signaling results and is indistinguishable from SCM-purified MSP by MALDI-TOF (*8*).

To verify that MSP is the signal for oocyte maturation and sheath contraction, we expressed and purified two MSP isoforms, MSP-77 and MSP-38, from *Escherichia coli* (*9*). Both isoforms, which differ by three amino acids, promote oocyte maturation and sheath contraction at rates equivalent to MSP

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