

lations from the actual estimates was found to be significantly greater than that of the randomized data ( $P = 0.017$ ), with the correlation for UPGMA being significantly less than the randomized value minimum ( $P < 0.011$ ). The heterogeneity of correlations among the set of four methods with UPGMA removed is no longer significantly large, but the correlation for parsimony is larger than the maximum of randomized values at  $P = 0.05$ . By these criteria, UPGMA appears to be significantly

worse than the other methods, and there is some evidence that parsimony is superior.

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## Chemical Signals from Host Plant and Sexual Behavior in a Moth

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In the phytophagous corn earworm, *Helicoverpa (Heliothis) zea*, females delay their reproductive behaviors until they find a suitable host on which to deposit their eggs. Perception of volatile chemical signals from corn silk triggers the production of sex pheromone followed by its release, which leads to mating. Several natural corn silk volatiles, including the plant hormone ethylene, induced pheromone production in *H. zea* females. Because *H. zea* larvae feed on the fruiting parts of a wide variety of hosts, ethylene, which is associated with fruit ripening, could act as a common cue.

**F**EMALES OF MOST SPECIES OF MOTHS use sex pheromones to attract their mates. Production of the pheromone in a number of moth species is controlled by a peptide hormone, the pheromone biosynthesis-activating neuropeptide (PBAN) (1). Release of PBAN in laboratory-reared females of the corn earworm, *Helicoverpa (Heliothis) zea*, is regulated primarily by photoperiod (2). However, in nature, photoperiodic control appears to be superceded by signals from the host plant (2). For instance, female progeny of *H. zea* collected from a corn field did not produce pheromone during scotophase until they encountered corn, one of the hosts for this insect (3). The presence of silk from an ear of corn was sufficient to elicit pheromone production followed by "calling" behavior or pheromone release (3). Moreover, physical contact between the females and corn silk was not required, which indicated the involvement of a volatile factor (or factors) produced by the host plant.

Several years ago, trans-2-hexenal, a volatile component of oak leaves, was implicated in the induction of sex pheromone production in the polyphemus moth, *Antheraea polyphemus* (4, 5). However, this phenomenon could not be confirmed in a subsequent study (6). Hence the signal (or signals) and the mechanism involved in the process have

remained a matter of conjecture. We show the efficacy of several chemical constituents of corn silk volatiles, including the plant hormone ethylene, in inducing pheromone production in *H. zea* females. Ethylene, widely used by plants in fruit ripening (7), may act as a common cue because *H. zea* larvae feed on the fruiting parts of a wide variety of hosts. Thus, the corn earworm female can recognize these plant chemicals and exploit this ability to coordinate its reproductive behavior with the availability of food for the offspring.

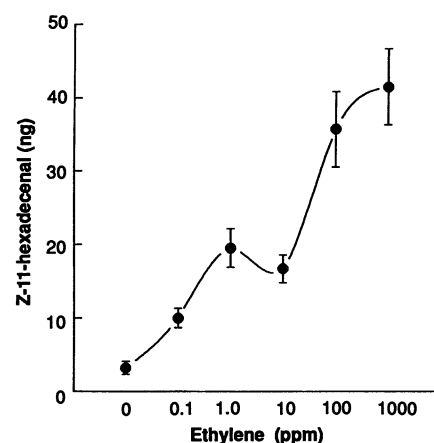
Larvae of *H. zea* were collected from corn fields (8) and reared through one generation on an artificial diet. The resulting females ( $F_1$ ) were used in all the experiments reported here. Pheromone titers [expressed as the quantity of its major component, (Z)-11-hexadecenal] were determined by capillary gas chromatography (9). The wild  $F_1$  females not exposed to corn produce only trace amounts of the pheromone ( $<3$  ng per female). However, in the presence of the plant host (corn silk), a 20- to 30-fold increase in pheromone production occurred (Table 1). Removal of corn silk before the onset of scotophase resulted in a much lower production of pheromone, showing that the presence of plant volatiles during scotophase is required for maximal induction. Pheromone production was also induced, although to varying degrees, when moths were provided with volatiles collected from corn silk, ether-soluble and water-soluble fractions of corn silk extracts, or an intact tomato fruit (also a host for *H. zea*) (10). These results show that wild females stand in contrast to moths reared in the laboratory

**Table 1.** Effect of host plant on sex pheromone production by wild  $F_1$  females of *H. zea*. Individual 2-day-old virgin females were placed in cylindrical plastic containers (10 cm tall, 5 cm in diameter, and 150 ml volume) with snap-on lids. Where indicated, corn silk (1 g) was placed under a Whatman filter paper at the bottom of the container for 18 hours (that is, until pheromone extractions were carried out) in corn silk-1. In 2, corn silk was removed before the onset of scotophase or 5 hours before extraction time. All means were significantly different at  $\alpha = 0.05$  (Dunn's test: distribution free multiple comparisons based on Kruskal-Wallis rank sums).

Treatment	n	Z-11-hexadecenal (ng/female $\pm$ SEM)
Control	10	2.1 $\pm$ 0.3
Corn silk-1	10	54.1 $\pm$ 6.4
Corn silk-2	5	11.1 $\pm$ 3.2

on artificial diet for many generations, which do not require host plant for the production of pheromone (2).

Because most species belonging to the *Heliothis-Helicoverpa* group feed on fruiting parts of various host plants and because the gaseous plant hormone ethylene is commonly produced by flowering plants, we tested the possibility that ethylene may act as a cue for pheromone production. First, using gas chromatography, we checked whether ethylene was a constituent of the volatiles produced by corn silk. On a fresh weight basis, a gram of corn silk produced  $2.07 \pm 0.47$  ( $n = 6$ ) and  $2.53 \pm 0.67$  ( $n = 6$ ) nl of ethylene per hour over a 24-hour period in two separate tests. We then determined the effect of exogenous ethylene on pheromone production in *H. zea* females. Ethylene at various concentrations was introduced into containers holding individual *H. zea* females in their second photophase. Pheromone was extracted during the following scotophase



**Fig. 1.** Pheromone production in *H. zea* wild  $F_1$  females as a function of ethylene concentration (mean  $\pm$  SEM,  $n = 7$ ).

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(after 18 hours) and quantified. As shown in Fig. 1, ethylene elicited concentration-dependent induction of pheromone production. Threshold levels for eliciting pheromone production were  $\leq 0.1$  ppm, consistent with that reported for many ethylene-mediated responses in higher plants (7, 11). Furthermore, this amount of ethylene is in the range of that produced by 1 g of corn silk in 18 hours ( $\sim 0.3$  ppm), suggesting that ethylene could account for part, though apparently not all, of the pheromonotropic activity of corn silk in our experiments. Therefore, we wanted to determine whether additional volatiles produced by corn silk might elicit higher levels of pheromone production.

Many volatiles from corn silk have been identified (12–14). We tested some of the most abundant and commercially available for pheromonotropic activity. Of the various compounds tested, 3-methyl-butan-1-ol and phenylacetaldehyde were the most effective in inducing pheromone production (Table 2). It is evident from the data in Table 2 that longer chain alcohols, which make up a major fraction of the volatiles from corn silk (12) and may be present in the mixture of volatiles from other host plants, could also contribute to the response of females to corn silk. These data raise the possibility that receptor systems in *H. zea* females for plant volatiles may mediate the pheromonotropic response. Additional evidence was sought to test this suggestion.

**Table 2.** Effect of various volatile chemicals from corn silk on pheromone production in wild *F<sub>1</sub>* females of *H. zea*. Whatman filter paper strips (1 by 6 cm) were treated with 1  $\mu$ g equivalent of the test chemical and suspended in the clear plastic container. The concentration of volatiles in the exposure chamber achieved from 1  $\mu$ g may be calculated for each chemical if 100% volatilization is assumed. These are, for instance, 3-methyl-butan-1-ol, 5.0 ppm; phenylacetaldehyde, 3.7 ppm. Individual 2-day-old virgin females were released into the containers during photophase. Control females were provided with a filter paper strip. One group of females was provided with 1 g of fresh corn silk placed under a filter paper disk at the bottom of the container. Pheromone was extracted after 18 hours (4 hours into the third scotophase) ( $n = 6$ ). Means followed by the same letter are not significantly different at  $\alpha = 0.05$  (statistical analysis method same as in Table 1).

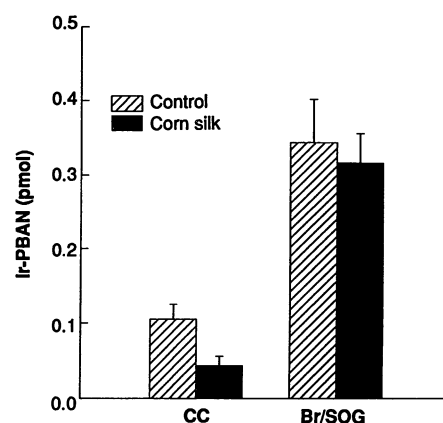
Test chemical	Z-11-hexadecenal (ng/female $\pm$ SEM)
Control	3.2 $\pm$ 1.3 <sup>d</sup>
Geraniol	6.4 $\pm$ 1.2 <sup>d</sup>
1-heptanol	12.9 $\pm$ 1.5 <sup>c</sup>
2-nonanol	21.7 $\pm$ 7.6 <sup>c</sup>
1-hexanol	35.1 $\pm$ 8.6 <sup>bc</sup>
2-methyl-butanal	40.3 $\pm$ 13.3 <sup>b</sup>
Phenylacetaldehyde	45.1 $\pm$ 13.3 <sup>ab</sup>
3-methyl-butan-1-ol	66.2 $\pm$ 10.0 <sup>a</sup>
Corn silk	63.0 $\pm$ 8.5 <sup>a</sup>

A common characteristic of ethylene responses in higher plants is their inhibition by silver ions (15); we would expect silver ions to be more generally disruptive of protein function, including that of insect chemoreceptors. Accordingly, we tested the effect of AgNO<sub>3</sub> on ethylene-induced pheromone production. Antennae (the principal olfactory organ in insects) of female moths were dipped in a solution of either 50  $\mu$ M silver nitrate or sodium nitrate, and the moths were then exposed to air alone or air containing 10 ppm of ethylene. The results (Table 3) show that silver nitrate treatment significantly reduced the response of female moths to ethylene, whereas the control moths treated with sodium nitrate responded normally. These findings reveal the presence of an ethylene-recognition system in female *H. zea*, thus supporting the involvement of ethylene in regulating pheromone production.

Although we show here that plant volatiles including the plant hormone ethylene induce pheromone production in female moths, the mechanism involved is not yet understood. The phenomenon described above is not universal to all phytophagous moths. Whereas some species require their host plant for mating and oviposition, others do not. An earlier report (3) suggested that the lack of pheromone production in wild *H. zea* could be due to an impairment of PBAN release, rather than its absence. Signals from the host plant, then, could induce the release of PBAN, leading to the production of the pheromone. The neurohemal corpora cardiaca, shown to contain immunoreactive PBAN [ir-PBAN; (16)], could reflect that release in a depletion of PBAN. Consistent with this view, we found that exposure to the host plant caused the loss of ir-PBAN from the corpora cardiaca

**Table 3.** Effect of blocking antennal receptors on pheromone production in wild *F<sub>1</sub>* females of *H. zea* in response to ethylene: Females were anaesthetized with CO<sub>2</sub> during their second photophase and then their antennae dipped for 1 min in solutions containing 50  $\mu$ M of test substance in 0.05% Tween 20. The females were then released in individual containers. Air or ethylene (10 ppm) was injected into these containers. Pheromone titer was determined after 18 hours. Control females were also anaesthetized. Means followed by the same letter are not significantly different at  $\alpha = 0.05$  (statistical analysis method same as in Table 1).

Treatment	<i>n</i>	Z-11-hexadecenal (ng/female $\pm$ SEM)
Control	5	5.7 $\pm$ 1.1 <sup>b</sup>
NaNO <sub>3</sub> , air	9	6.8 $\pm$ 1.2 <sup>b</sup>
NaNO <sub>3</sub> , ethylene	9	28.5 $\pm$ 3.9 <sup>a</sup>
AgNO <sub>3</sub> , air	9	11.5 $\pm$ 1.3 <sup>b</sup>
AgNO <sub>3</sub> , ethylene	9	15.7 $\pm$ 2.7 <sup>b</sup>



**Fig. 2.** Immunoreactive-PBAN (Ir-PBAN) in brain-subesophageal ganglion (Br-SOG, site of PBAN production) and corpora cardiaca (CC, putative site of PBAN release) complexes of *H. zea* wild *F<sub>1</sub>* females after exposure to corn silk during scotophase. Peptide was quantified by competitive ELISA (16, 17). Bars indicate SEM ( $n = 9$ ). The amount of ir-PBAN in CC of females exposed to corn silk is significantly lower than in controls ( $P = 0.022$  by unpaired *t* test).

(Fig. 2), apparently without affecting ir-PBAN in the brain-subesophageal ganglion, known to contain the PBAN-producing neurosecretory cells (16).

*Helicoverpa zea* infest wild hosts such as geranium early in the season (18), before moving to cultivated hosts such as corn and cotton. We suspect that at least some of the volatile compounds found in corn silk are either common to all hosts or that the moths are able to respond to a variety of compounds from different plant hosts. Ethylene would only act as an indicator of the host-plants' maturity. A recently characterized class of general odorant-binding proteins from antennae of several species of female moths shows a 95% conservation of their NH<sub>2</sub>-terminal sequences (19). Such proteins may participate in the pheromonotropic response by conveying an array of plant volatiles to their receptors in the antennae.

Virgin female moths can delay egg laying for several days but mating causes a switch to ovipositional behavior and the females soon begin to lay eggs. Therefore, to ensure the survival of their progeny on a suitable host plant, the female moths locate a host prior to mating (20). The action of host plant volatiles, including the plant hormone ethylene, in regulating production of sex pheromone in a pest insect is illustrative of the intricate mechanisms that have evolved in nature to enable moths to coordinate their reproductive behavior with the availability of food for their progeny.

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## Ordered Self-Assembly of Polypeptide Fragments to Form Nativelike Dimeric *trp* Repressor

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Subdomain-size proteolytic fragments of *Escherichia coli* *trp* repressor have been produced that assemble in defined order to regenerate fully native dimers. By characterization of the secondary and tertiary structures of isolated and recombined fragments, the structure of assembly intermediates can be correlated with the kinetic folding pathway of the intact repressor deduced from spectroscopic measurement of folding rates. The nativelike structure of these intermediates provides further evidence that protein folding pathways reflect the stabilities of secondary structural units and assemblies found in the native state. The proteolytic method should be generally useful in adding structural detail to spectroscopically determined folding mechanisms.

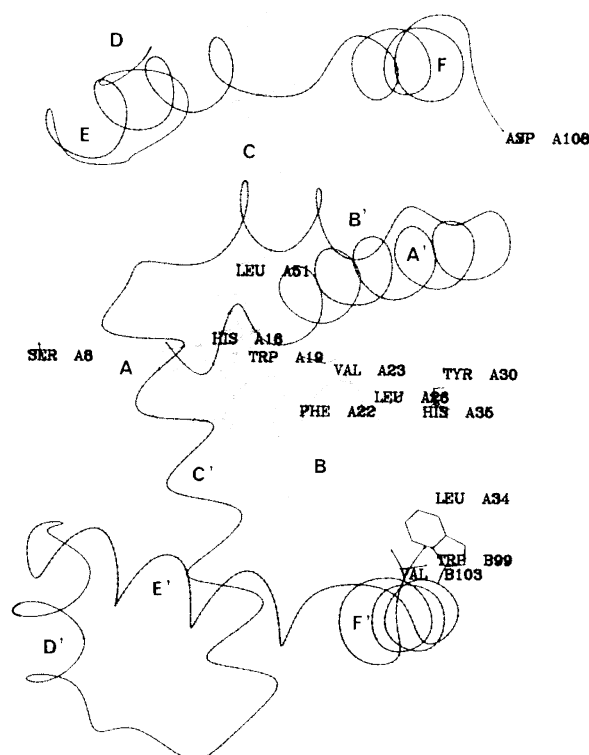
A MAJOR GOAL OF STUDYING PROTEIN folding mechanisms is to describe the structures of intermediates along the folding pathway [for review, see (1)]. Only one method has the required temporal and structural resolution to follow the folding process: nuclear magnetic resonance (NMR) analysis of hydrogen exchange rates for individual protons (2). Although this method is powerful, its application is limited to the rather small set of proteins under ~20 kD for which complete resonance assignments are available. More widely applicable kinetic studies that use global spectroscopic probes, such as near-ultraviolet (UV) extinction coefficient, far-UV ellipticity, or fluorescence intensity, yield only formal mechanistic intermediates that lack structural definition. It would be generally useful if such abstract mechanisms could be fleshed out in structural detail. We report (i) an approach that uses biochemical dissection of a complex protein into subdomain-size fragments and (ii) structural characterization of the ordered assembly process that these fragments undergo (3).

The *trp* aporepressor of *Escherichia coli*

(TrpR) has an unusually complex subunit architecture (4). The dimer is built from two

highly intertwined chains (Fig. 1), each of which has an overall dumbbell-shaped fold similar to that of calmodulin or troponin (5). Helices A, B, and part of C, together with A', B', and part of C', constitute most of the subunit interface. These six helices contain many of the hydrophobic residues of the protein, are tightly packed, and show little flexibility (6–8). The amphipathic COOH-terminal helix, F, from one subunit packs against helices A' and B' from the other subunit to complete the dimer interface, creating a complex topology. Together, helices A, B, F, and part of C from each subunit form a rigid core domain (4, 6), whereas helices D and E are involved in DNA binding (9). The intertwined nature of the TrpR dimer implies that association

**Fig. 1.** Structure of the *trp* repressor dimer. The polypeptide backbone is represented by a string from residues 8 to 108. Residues 8 to 71 (N-fragment) of subunit A are shown in green, and those from subunit B are in purple; residues 72 to 108 (C-fragment) from each subunit are shown in blue. Helices are labeled A to F in subunit A and A' to F' in subunit B. Nativelike packing of certain hydrophobic residues is detectable by NMR. The side chains of these residues are shown in skeletal form and identified by three-letter code, residue number, and subunit (A or B); symmetry-related residues from the two subunits are indicated only once. The model was constructed with coordinates from file 3WRP [TrpR holorepressor (4, 6)] from the Brookhaven Protein Data Bank with deletion of the exogenous Trp ligand.



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