Fungal Ecdysteroid-22-oxidase, a New Tool for Manipulating Ecdysteroid Signaling and Insect Development*1†‡

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Steroid hormones ecdysteroids regulate varieties of developmental processes in insects. Although the ecdysteroid titer can be increased experimentally with ease, its artificial reduction, although desirable, is very difficult to achieve. Here we characterized the ecdysteroid-inactivating enzyme ecdysteroid-22-oxidase (E22O) from the entomopathogenic fungus Nomuraea rileyi and used it to develop methods for reducing ecdysteroid titer and thereby controlling insect development. Kcat and Kcat values of the purified E22O for oxidizing ecdysone were 4.4 μM and 8.4/s, respectively, indicating that E22O can inactivate ecdysone more efficiently than other ecdysteroid inactivating enzymes characterized so far. The cloned E22O cDNA encoded a FAD-dependent oxidoreductase. Injection of recombinant E22O into the silkworm Bombyx mori interfered with larval molting and metamorphosis. In the hemolymph of E22O-injected pupae, the titer of hormonally active 20-hydroxyecdysone decreased and concomitantly large amounts of inactive 22-dehydroecdysteroids accumulated. E22O injection also prevented molting of various other insects. In the larvae of the crab spider Haritalodes basipunctalis, E22O injection induced a diapause-like developmental arrest, which, as in normal diapause, was broken by chilling. Transient expression of the E22O gene by in vivo lipofection effectively decreased the 20-hydroxyecdysone titer and blocked molting in B. mori. Transgenic expression of E22O in Drosophila melanogaster caused embryonic morphological defects, phenotypes of which were very similar to those of the ecdysteroid synthesis deficient mutants. Thus, as the first available simple but versatile tool for reducing the internal ecdysteroid titer, E22O could find use in controlling a broad range of ecdysteroid-associated developmental and physiological phenomena.

In insects, the steroid hormones ecdysteroids, primarily 20-hydroxyecdysone (20E), play important roles in the regulation of various developmental and physiological processes such as embryogenesis, molting, metamorphosis, reproduction, and diapause (1–4). Titters of ecdysteroids are precisely controlled by a combination of synthetic reactions, most of which proceed in the prothoracic gland, whereas they are inactivated in various peripheral organs (5). In the last decade, eight ecdysteroid synthesis enzymes were identified, mainly from the fruit fly Drosophila melanogaster and silkworm Bombyx mori (6–16). Five of these enzymes were cytochrome P450 encoded by the so-called Halloween genes of D. melanogaster and mutants of which were all embryonic lethal (17). In contrast, the ecdysteroid inactivation process has received relatively less attention. So far, four ecdysteroid inactivation enzymes have been identified in insects (18–23). Mutations in or knockdown of those genes interfered with insect metamorphoses (21–23), indicating that both synthesis and inactivation of ecdysteroids are essential for the normal development of insects.

Some insect pathogens also use ecdysteroid inactivation enzymes. The best-known example is the baculoviruses, which express the ecdysteroid UDP-glucosyltransferase that inactivates ecdysteroids via sugar conjugation at position C22 (24). Another example is the entomopathogenic fungus Nomuraea rileyi that secretes the ecdysteroid-22-oxidase N. rileyi.

The abbreviations used are: 20E, 20-hydroxyecdysone; E22O, ecdysteroid-22-oxidase.
researching a variety of ecdysteroid-dependent phenomena, the ecdysteroid titer, could serve as a powerful tool for furthering our understanding of hormonal functions and modes of action of this hormone in a number of insect species (1–4). A reduction in the ecdysteroid titer of insect species (1–4) is a sufficient endocrinological stimulus to induce diapause-like developmental arrest in the crambid Tenebrio molitor was purchased from a pet shop and reared on powdered bird food. These insects were reared at 25 °C under a 12-h light, 12-h dark (12L:12D) photoperiod. H. basipunctalis was collected in Tsukuba and reared on Firmiana simplex leaves at 25 °C under 16L:8D photoperiod (nondiapausing condition) or at 17 °C under 8L:16D photoperiod (diapause-inducing condition). D. melanogaster were reared on a standard agar-cornmeal medium at 25 °C under 12L:12D photoperiod. Mutant strain hsp70-GAL4 was obtained from the Drosophila Genetic Resource Center at Kyoto Institute of Technology. Mutant strains engrailed-GAL4 and Actin5C-GAL4 were obtained from the Bloomington Stock Center. The Actin5C-GAL4 strain was originally established by Dr. Yasushi Hiromi at the National Institute for Genetics, Japan. N. rileyi, maintained at the National Institute of Agrobioscience, was cultured in a medium containing 20 g of maltose, 5 g of tryptone, and 5 g of yeast extract in 1 liter of water. Conditioned media for N. rileyi were prepared as described previously (25).

Ecdysteroids and Brassinosteroids—Ecdysone (Sigma) and 20-hydroxyecdysone (Mitaka Pharmaceutical Co.) were purified using HPLC (LC-10AT, Shimadzu). Each ecdysteroid was applied to a C18 reverse-phase column (TSK gel ODS-80Ts, 25 × 200 mm, TOSOH) and then eluted with a 20–30% linear gradient of acetonitrile using a flow rate of 0.6 ml/min. Fractions corresponding to each ecdysteroid were pooled, methanol was evaporated off, and dried ecdysteroid was weighed and then dissolved in ethanol. 22-Dehydroecdysone and 22-dehydro-20-hydroxyecdysone were synthesized by reacting ec dysone and 20-hydroxyecdysone, respectively, with the conditioned media of Sf9-E22O cells. They were then purified by HPLC as above, weighed, and dissolved in ethanol. HPLC analysis confirmed that these ecdysteroids were >99% pure. Ponasterone A (Invitrogen), brassinolide (Wako), and castasterone (Wako) were dissolved in ethanol. The ecdysteroid and brassinosteroid solutions were diluted with distilled water and reacted with E22O-containing solutions.

E22O Activity Assay—An aliquot of E22O-containing solution was mixed with an equal volume of 200 μM ecdysone and incubated at 25 °C. Ten min later, a double volume of ethanol was added to stop the reaction and the mixture was centrifuged at 18,000 × g for 10 min. Ecdysone and synthesized 22-dehydroec dysone remaining in the supernatant were separated by HPLC and the amount of 22-dehydroecdysone was calculated from the ratios of peak areas of the two ecdysteroids. Km and Vmax values were calculated using the GraphPad Prism 5 program (GraphPad Software).

Purification of E22O—N. rileyi-conditioned medium (152 ml) was mixed with a protease inhibitor mixture and solid ammonium sulfate was then added to achieve 50% saturation,
and the mixture was continuously stirred at 4 °C overnight. After centrifugation at 10,000 × g for 20 min, E22O was purified from the resulting supernatant by HPLC (Model Bio-HPLC system, TOSOH). The crude extract was applied to a HiTrap Phenyl-Sepharose HP column (Amersham Biosciences) equilibrated with buffer A (20 mM Tris-HCl (pH 7.6)) containing 50% ammonium sulfate. The column was eluted using a linear gradient (0–50%) of ammonium sulfate in buffer A. Fractions containing E22O were pooled, dialyzed against buffer A containing 0.3 M NaCl, and concentrated using an Ultrafree Biomax-5 Centrifugal filter. The concentrated E22O was further purified using a HiTrap Q column (Amersham Biosciences). Fractions containing E22O were pooled, dialyzed against the Ultrafree Biomax-5 Centrifugal filter again. The concentrated E22O was further purified using a HiTrap Q column (Amersham Biosciences). A partial cDNA was obtained from the N. rileyi mycelia using TRIzol (Invitrogen) and reverse-transcribed using Ready-To-Go T-Prime First-strand Beads (Amersham Biosciences). A partial E22O cDNA was cloned from the cDNA pool by PCR. Forward and reverse PCR primers were designed on the basis of the sequences of the purified E22O and its limited V8-proteolysis product, respectively. The first PCR was carried out using the E22O-dF1 (5′-TICCCARGGIGGITGYAG-3′) and E22O-dR1 (5′-CAIGCITITTITIACTRTRTG-3′) primers. The second nested PCR was carried out using the E22O-dF1 (5′-TICCCARGGIGGITGYAG-3′) and E22O-dF1 (5′-TICCCARGGIGGITGYAG-3′) primers. The full-length E22O cDNA was obtained by combining 5′-RACE and 3′-RACE reactions with primers that were predicted using the SignalP 3.0 program (27). The full-length E22O cDNA was sequenced by using SMART RACE cDNA Amplification Kit (Clontech). The nucleotide sequence of the full-length E22O cDNA was deposited in the GenBank™/EMBL/DDBJ databases (accession number AB675078). A putative secretion signal of E22O was predicted using the SignalP 3.0 program (27). The full-length E22O cDNA was cloned into the EcoRI/BglII site of the pUAST vector (29). The embryos used as recipients for DNA injection to generate transgenic lines were yellow white (yac w11b) flies. Transgenic flies carrying UAS-E22O constructs (UAS-E22O line) was injected into the hemocoel of insects using a microsyringe (Hamilton) with a 31-gauge point-4 needle.

Transgenic Expression of E22O Gene in Fruit Fly—The entire coding region of E22O cDNA from plpZT-E22O was cloned into the EcoRI/BglII site of the pUAST vector (29). The embryos used as recipients for DNA injection to generate transgenic lines were yellow white (yac w11b) flies. Transgenic flies carrying UAS-E22O constructs (UAS-E22O line) were generated as described previously (30). The UAS-E22O line was crossed with different GAL4 driver lines and F1 individuals were used for experiments.

Quantification of Ecdysteroid Titer—Hemolymph samples were individually collected from B. mori pupa or H. basipunctalis larvae, mixed vigorously with a triple volume of methanol, and centrifuged at 18,000 × g for 5 min. D. melanogaster embryos were collected as batches 7–12 h after egg laying, a time when embryonic ecdysteroid titer is the highest (31), homogenized in methanol, and then centrifuged. Total ecdysteroid content in the supernatant of these samples were measured by radioimmunoassay using 20E as a standard (32). Supernatant from the B. mori sample was run on HPLC and a fraction containing ecysone, 20E, 22-dehydroecdysone, or 22-dehydro-20-hydroxyecdysone was collected separately. The fractions were dried, dissolved in methanol again, and subjected to radioimmunoassay using each ecdysteroid as a standard. The affinities of the antibody used in radioimmunoassay to ecysone, 20E, 22-dehydroecdysone, and 22-dehydro-20-hydroxyecdysone were ~1:30:3:1.
values were the same (i.e. 4.3 μM) for both ecdysteroids (Fig. 2F). This observed $K_{cat}$ of the purified E22O for ecdysone was much higher than those of the ecdysteroid UDP-glucosyltransferase of the *Autographa californica* nucleopolyhedrovirus ($K_{cat} = 0.069/s$) and ecdysone oxidase of the cotton leafworm *Spodoptera littoralis* ($K_{cat} = 0.11–0.12/s$, calculated assuming that the molecular mass of the enzyme is 190 kDa), whereas the $K_m$ values of all three enzymes for ecdysone were similar (19, 33). These results suggest that E22O inactivates ecdysone much more efficiently than the two other well characterized ecdysteroid inactivation enzymes. E22O also oxidized and inactivated other ecdysteroids that contain the hydroxyl group at C22, for example, ponasterone A, however, had no effect on plant steroid hormones brassinosteroids, such as brassinolide and castasterone, even though they also have the hydroxyl group at C22 (data not shown).

Next, to clone the E22O cDNA, we first sequenced the N termini of the purified E22O protein and one of the peptides produced by limited hydrolysis of E22O using V8 protease (supplemental Fig. S3). Using degenerated primers, designed on the basis of these amino acid sequences, a partial E22O cDNA fragment was cloned by RT-PCR. The 5′ and 3′ ends of the E22O cDNA were then cloned using the 5′- and 3′-RACE techniques. The full-length E22O cDNA encoded a novel FAD-binding oxidoreductase comprised of 594 amino acids (supplemental Fig. S3). Consistent with the prediction that E22O is a flavoprotein, purified E22O had a brownish color with an absorbance at 454 nm. The N-terminal end of E22O contained a putative signal peptide, and the amino acid sequence following it matched the N-terminal sequence of the purified protein as determined above, suggesting that E22O is processed after the signal peptide and secreted from the cells. The amino acid sequence of E22O was up to 55% identical to those of the alcohol oxidases identified from various fungi (supplemental Fig. S3). Although some of these oxidases are involved in the biosynthesis of biologically active agents, such as *Hypomyces subiculosus* alcohol oxidase involved in hypothemycin synthesis and *Fusarium incarnatum* AP59 in apicidin synthesis (34, 35), none of them are known to be involved in the modification of steroids or hormones.

### Activity of Recombinant E22O

When the E22O cDNA was transiently expressed in S9 cells, high ecdysone-oxidizing activity was found in the culture media (supplemental Fig. S1A). Remarkably, 50 μl of the media completely oxidized an equal volume of 200 μM (100 μM final concentration) ecdysone within 10 min (supplemental Fig. S1B); this final concentration of ecdysone was 100 times higher than the maximal titer found in the *B. mori* pupal hemolymph (Fig. 5C). This activity was comparable with or even higher than that observed in the *N. rileyi* conditioned media. In contrast, hardly any activity was observed in the cellular lysates (supplemental Fig. S1A). These results confirmed that the cloned cDNA encodes E22O and that E22O is a secretory protein.

Next, we generated an S9 cell line that stably expressed the E22O cDNA (S9-E22O cell line) and injected its conditioned medium into *B. mori* individuals to test whether it would decrease the hemolymph ecdysteroid titer and affect their growth. We have previously shown that injection of *N. rileyi*-conditioned medium into the midpuparia instar larvae of
B. mori induced precocious pupation (25). Consistent with this observation, some of the larvae that were injected with Sf9-E22O-conditioned medium at the beginning of the penultimate instar ate food 3–4 days longer than the control, started spinning a week later and half of them pupated precociously. When the conditioned medium was injected into the late-penultimate instar larvae, the last larval ecdysis was inhibited (Fig. 3B). Injection of Sf9-E22O-conditioned medium into B. mori pupae 2 days after pupation prolonged the pupal period and interfered with adult emergence (Fig. 3C and D). In the hemolymph of the E22O-injected pupae, the 20E titer decreased and instead large amounts of 22-dehydroecdysone and 22-dehydro-20-hydroxyecdysone were present on day 4 when the 20E titer is the highest in the control (Figs. 3E and 5C). These results indicate that the conditioned media of Sf9-E22O cells can also be used to reduce the 20E titer and thereby manipulate the growth of B. mori.

Molt Inhibition by E22O in Various Insects—We next examined how E22O would affect the developmental programs in insects other than B. mori. E22O-injected last instar larvae of a geometrid moth Naxa seriaria (Lepidoptera) remained as larvae for a much longer period than the controls, and eventually died without initiating pupation (Fig. 4, A and B). Similar results were obtained with the last instar larvae of the blowfly L. seri-
cata (Diptera) and bean bug R. clavatus (Hemiptera). In both species, the larval-pupal or nymphal-adult metamorphosis was rarely observed after E22O injection, whereas larvae or nymphs injected with the control medium completed metamorphosis within 2 weeks (Fig. 4, C-F). Injection of E22O into the penultimate or last instar larvae of the yellow mealworm T. molitor (Coleoptera) completely suppressed larval molting and 70% of them died as pharate pupae (Table 1). Furthermore, 70% of E22O-injected prepupae died without completing pupation. E22O injection into T. molitor pupae interfered with the normal adult eclosion and many deformed adults with folded or heavily curled wings emerged. Thus, E22O blocked molting and metamorphosis of 4 additional species belonging to different orders.

Transient Expression of E22O Gene in Silkworm—We have recently established an in vivo lipofection method to express foreign genes in B. mori larvae (28). Using this technique, the E22O gene was expressed in B. mori 3rd instar larvae. Four days after the lipofection, 0.098 pmol/l/g/d (46 ng/ml/min, n = 2) of ecdysone-oxidizing activity was observed in the plasma of the E22O-expressing larvae, indicating that the E22O gene was successfully expressed in larval tissues and expressed E22O protein was secreted into hemolymph. The E22O-expressing larvae continued eating in the instar and grew much bigger than the control 3rd instar larvae (Table 2 and supplemental Fig. S4A).

### TABLE 1
Effects of injection of Sf9-E22O conditioned medium into T. molitor

| Injected stage | Injected medium | n  | Total number of larval molts | Dead larva | Dead prepupa | Unsuccessful pupation | Dead pupa | Dead pharate adult | Unsuccessful eclosion | Deformed adult | Normal adult |
|----------------|-----------------|----|-------------------------------|------------|--------------|-----------------------|-----------|--------------------|----------------------|---------------|-------------|
| Larvae         | Control medium  | 84 | 45                            | 33         | 0            | 2                     | 0         | 0                  | 0                    | 64            |             |
|                | E22O medium     | 83 | 0                             | 27         | 43           | 28                    | 0         | 0                  | 0                    | 2             |             |
| Preparpae      | Control medium  | 111| 3                             | 8          | 21           | 3                     | 1         | 0                  | 0                    | 86            |             |
|                | E22O medium     | 79 | 49                            | 24         | 8            | 4                     | 8         | 1                  | 14                   | 0             |             |
| Pupae          | Control medium  | 43 | 2                             | 0          | 5            | 2                     | 0         | 0                  | 0                    | 93            |             |
|                | E22O medium     | 79 | 13                            | 10         | 0            | 13                    | 10        | 0                  | 73                   | 4             |             |

* Individuals that died within 3 days of the injection were excluded from data.

* This category includes adults with folded or heavily curled wings.

* This includes both penultimate and last instar larvae.

### TABLE 2
Effects of E22O gene expression on the growth of B. mori larvae

The E22O cDNA was introduced into B. mori by in vivo lipofection at the beginning of 3rd larval instar, and larval growth was subsequently monitored.

| Plasmid     | n     | Average 3rd instar period (day)* | Death during 3rd instar | Death during 4th instar | Precocious spinning | Ecdysis into last instar |
|-------------|-------|----------------------------------|-------------------------|-------------------------|---------------------|--------------------------|
|             |       |                                  | Larval death (1–5 days after injection) | Larval death (6–10 days after injection) | Larval death (>10 days after injection) | Unsuccessful eclosion | Unsuccessful eclosion | %% | %% |
| Control plasmid | 110   | 4.7                               | 1                       | 0                        | 0                   | 0                        | 5                  | 1       | 0 | 93 |
| pE220         | 110   | 7.1                               | 2                       | 0                        | 19                  | 6                        | 25                 | 2       | 7 | 34 |

* Only larvae that molted normally into the 4th instar were included in the calculation. The two values are significantly different (p < 0.01) by Student’s t test.
the control 4th instar larvae (supplemental Fig. S4B). Similar results were obtained by expressing E22O in the 4th instar larvae (supplemental Fig. S4C).

Next, the E22O-expressing plasmid was lipofected into the spinning 4th instar larvae. Whereas most of these larvae pupated normally, 85% of the pupae could not complete the pupal-adult metamorphosis (Fig. 5A). Half of these pupae grew to the pharate adult but could not eclose from the pupal case normally (supplemental Fig. S4D).

We compared the ecdysteroid titers in the hemolymph of the E22O-expressing and control pupae. In controls, the total ecdysteroid titer increased after pupation, reached a peak (~5 μg/ml) on day 4, and then decreased to the basal level (Fig. 5B); the ecdysone and 20E titers on the other hand peaked at around 500 ng/ml on days 2–3 and 4, respectively, and then decreased rapidly (Fig. 5C). These temporal changes of total ecdysteroid, ecdysone, and 20E were similar to those observed in Manduca sexta pupae (36). The sum of the ecdysone and 20E titers was 800 ng/ml at the maximum, suggesting that the hemolymph contained much more amounts of other ecdysteroids that could react with the antibody used in RIA. In contrast, the total ecdysteroid titer continued to increase in the hemolymph of the E22O-expressing pupae (Fig. 5B). The maximal ecdysone titer (~400 ng/ml) was observed on day 2, as in the controls, but thereafter the titer maintained a level of more than 100 ng/ml (Fig. 5D). The 20E titer did not show any obvious peak, but 100–300 ng/ml of 20E was present throughout the pupal period. The maximal 20E titer in the E22O-expressing pupae was thus half of that in the controls. Although 22-dehydroecdysone and 22-dehydro-20-hydroxyecdysone were not detected in the controls, large amounts of 22-dehydroecdysteroids, particularly 22-dehydroecdysone, accumulated in the hemolymph of the E22O-expressing pupae (Fig. 5D). This affinity of the antibody used in RIA was 10 times lower to 22-dehydroecdysone than to 20E (see “Experimental Procedures”) and therefore the contribution of 22-dehydroecdysone expressed in the 20E equivalent was expected to be very small, suggesting that large amounts of unidentified ecdysteroids were also present in the hemolymph of the E22O-expressing pupae. Taken together, these results indicate that transient expression of E22O dramatically altered the temporal patterns of the ecdysteroid titers, and affected both larval molting and metamorphosis in B. mori. An injection of purified 22-dehydroecdysone or 22-dehydro-20-hydroxyecdysone into late pupae had no effects (data not shown), suggesting reduction of 20E at the peak time or its sustained presence, not accumulation of those 22-dehydroecdysteroids, caused the developmental abnormalities.

**Transgenic Expression of E22O Gene in Fruit Fly**—We also examined the effects of overexpression of the E22O gene in the fruit fly D. melanogaster using the GAL4/UAS gene expression system (29). When the UAS-E22O line was crossed with the engrailed-GAL4 line, most (99.8%) of the F1 individuals were embryonic lethal. They developed to around stage 14 and completed segmentation, but then stopped further differentiation. The denticle belt was not formed in 75% of them (Fig. 6A). Similar results were obtained when the UAS-E22O line was crossed with the hsp70-GAL4 line even without heat shock or crossed with the Actin5C-GAL4 line (data not shown). These phenotypes were very similar to those of the ecdysteroid biosynthesis-deficient mutants of Drosophila, such as disembodied, shadow, phantom, spook, and shroud (17), suggesting that the ecdysteroid contents in the E22O-expressing embryos were reduced. In fact, at the peak time of the embryonic ecdysteroid titer in wild type (7–12 h after egg laying, corresponding to stages 12–15) (31), the total ecdysteroid titer in the engrailed-GAL4>UAS-E22O embryos was around half that of wild type embryos (Fig. 6B).

**Induction of Diapause in H. basipunctalis by E22O Injection**—Although it is widely accepted that low ecdysteroid titer is important for the maintenance of larval and pupal diapause (4), it is still unclear whether a reduction of ecdysteroid titer is sufficient to
Effects of transgenic E22O expression in D. melanogaster embryo. A, a typical phenotype of engrailed-GAL4>UAS-E22O embryo. Left, wild type; right, engrailed-GAL4>UAS-E22O. Note that even though the segmentation was completed, the denticle belt was not formed in the transgenic fly. B, comparison of the total ecdysteroid titer (20E equivalent) between the wild type and engrailed-GAL4>UAS-E22O embryos. The ecdysteroid titer was measured in batches of eggs collected 7–12 h after the oviposition. Error bars represent S.E. (n = 6–8). Asterisks (**) indicate that the values are significantly different (p < 0.01) by Student’s t test.

induce diapause. To address this question, we examined the effects of E22O injection on larval diapause of the crambid moth \textit{H. basipunctalis}.

\textit{H. basipunctalis} larvae entered diapause in the last larval instar after the wandering behavior when reared at 17 °C under a short day length (diapausa-inducing condition), whereas they pupated and then emerged as adults when reared at 25 °C under a long day length (nondiapausing condition) (Fig. 7, A and F). First, we verified that the hemolymph ecdysteroid titer is kept low in the diapausing \textit{H. basipunctalis} larvae. In the nondiapausing last instar larvae, the total ecdysteroid was less than 20 ng/ml during the feeding period (Fig. 7L). It increased drastically after the wandering behavior and peaked at around 600 ng/ml 1 day before pupation. In the diapausing last instar larvae, the ecdysteroid titer was less than 20 ng/ml during the feeding period as in the nondiapausing larvae (Fig. 7L). It increased slightly after the wandering behavior but did not exceed 40 ng/ml for the subsequent 4 weeks. Thus, the hemolymph ecdysteroid titer was kept at a much lower level in the diapausing larvae than in the nondiapausing larvae as expected. Those differences in the ecdysteroid titers between the nondiapausing and diapausing \textit{H. basipunctalis} larvae were very similar to those observed in other insects that enter diapause facultatively, such as larvae of \textit{Pimpla instigator} (37) and \textit{Ostrinia nubilalis} (38, 39) and pupae of \textit{M. sexta} (40), \textit{Sarcophaga argyrostoma} (41), \textit{Boettcherisca peregrina} (42), and \textit{Mamestra brasicae} (43).

When the conditioned medium of Sf9-E22O cells was injected into the nondiapausing last instar larvae 1 day after the wandering behavior, pupation was delayed and some individu-als remained at the larval stage for very long periods (Fig. 7B). When those larvae were transferred to the diapause-inducing condition or kept at 5 °C, in both cases most of them lived longer than 2 months as larvae without eating anything (Fig. 7, E and H). These developmental characteristics were very similar to those of the diapause-destined larvae (Fig. 7, C, F, and I) but distinct from those of the intact nondiapausing larvae (Fig. 7, A, D, and G). Particularly, all of intact nondiapausing larvae transferred to 5 °C died within 2 months, indicating that E22O injection rapidly imparted high cold hardiness to \textit{H. basipunctalis} larvae.

When the E22O-injected larvae were reared under diapause-breaking conditions (5 °C for 6 weeks and then transferred to diapause-inducing condition), they pupated and then emerged as adults, as did diapausing larvae (Fig. 5, J and K). The E22O-injected nondiapausing larvae were thus in a physiologically similar state to that of the diapausing larvae, which strongly suggest that E22O injection induced diapause or a diapause-like state in the \textit{N. basipunctalis} larvae. As far as we know, these results are the first evidence indicating that a reduction in the ecdysteroid titer is a sufficient endocrinological stimulus to induce diapause in insects.

DISCUSSION

We have characterized the ecdysteroid inactivation enzyme E22O from an entomopathogenic fungus \textit{N. rileyi} and have shown that both injection of the recombinant E22O protein and forced expression of the \textit{E22O} gene reduce the internal ecdysteroid titer and affect the development and physiology of several insect taxa. The E22O-modified phenomena included embryogenesis, larval–larval molt, larval–pupal metamorphosis, pupal- or nymphal–adult metamorphosis, and diapause. Thus, E22O influenced most of the major ecdysteroid-regulated events during insect development.

Injection of recombinant E22O protein or transient expression of the \textit{E22O} gene inhibited molting and metamorphosis in various insect species. In \textit{N. seriaria}, \textit{L. sericata}, and \textit{R. clavatus}, the E22O-injected last instar larvae or nymphs remained at the stage they were injected for a long period until they died without initiating molt. It is most likely that the injected E22O maintained the titers of ecdysteroids in the hemolymph, particularly that of 20E, below the thresholds to induce metamorphosis in these species. In contrast, more than half of E22O-injected \textit{B. mori} and \textit{T. molitor} larvae eventually initiated molting responses although many of them could not complete the molting, suggesting that E22O suppressed the rise of ecdysteroid titer incompletely or changed its temporal profile and thereby interfered with molting and metamorphosis. In fact, a lower but significant level of 20E was continuously present in \textit{B. mori} pupae that were forced to express the E22O gene. These differences imply that detailed mechanisms underlying the molt inhibition caused by E22O may vary according to the insect species.

Reduction of ecdysteroid titers either by injection of E22O protein or by expression of the \textit{E22O} gene induced precocious metamorphosis in \textit{B. mori}. These results may appear strange because precocious metamorphosis is widely known to be a typical phenotype of the lack of juvenile hormone (1). However,
it was also reported that the *B. mori* 4th instar larvae, in which the ecdysteroid synthesis was suppressed by using the imidazole compound KK-42, pupated precociously (44). In addition, there are some reports showing that the ecdysteroid deficiency causes precocious metamorphosis also in *D. melanogaster* (45–47). The precocious pupation in *D. melanogaster*, induced as a result of lowered ecdysteroid titer, was explained as follows: due to the low ecdysteroid titer, the penultimate instar larvae were unable to initiate the last larval-larval molt and kept on feeding, and consequently grew to the point where they surpassed the

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**FIGURE 7. Growth, survival, and ecdysteroid titer of *H. basipunctalis* larvae under different conditions.** Nondiapausing larvae reared under nondiapausing conditions (A, D, and G), nondiapausing larvae injected with 30 μl of the conditioned culture medium of the Sf9-E22O cells 1 day after wandering (B, E, and H), and diapause-destined larvae reared under diapause-inducing conditions (C, F, and I) were reared under nondiapausing conditions (A-C), diapause-inducing conditions (D-F), or at 5 °C (G-I) after wandering, and their subsequent growth and survival were monitored. The E22O-injected nondiapausing larvae (J) and intact diapause-destined larvae (K) were reared also under diapause-breaking conditions and their growth was monitored. Sixty to 300 larvae were used for each experiment. L, changes in the total ecdysteroid titer (20E equivalent) in the hemolymph of nondiapausing and diapausing last instar larvae. Larvae were reared under the nondiapausing and diapause-inducing conditions continuously. Their growth are shown in A and F, respectively. *Error bars* represent S.E. (*n* = 2–17).
larvae was also prolonged, a similar mechanism may underlie in (46). Because the 4th larval period of the E22O-treated B. mori larvae was also prolonged, a similar mechanism may underlie in the induction of precocious pupation in B. mori.

Among the multiple applications of E22O tested here, transgenic insects carrying the E22O gene seem to be an ideal system for controlling the ecdysteroid titer at any stage of the development. The E22O-expressing transgenic D. melanogaster were, however, all embryonic lethal irrespective of the GAL4 driver lines used. These results were in contrast to those of the ecdysteroid 26-hydroxylase (cyt18A1)-expressing flies, where the time of death changed from the embryonic stage to the last larval instar stage depending on the GAL4 driver line used for crossing (21). Thus, contrary to our expectation, the E22O gene appears too strong to use in transgenic flies, suggesting that mutatons to moderate the enzymatic activity of E22O may be necessary for its transgenic use.

Using E22O, we not only confirmed the importance of ecdysteroids in embryogenesis, larval molting, and metamorphoses, but also answered an unsolved question associated with diapause. E22O-injected H. basipunctalis last instar larvae remained as larvae for a long period as found with N. seriaria, L. sericata, and R. clavatus. We assumed that this was not a simple developmental arrest, but an artificially induced diapause or a diapause-like state based on the following three reasons. First, the E22O-injected H. basipunctalis larvae survived much longer than the above three species without eating anything. Second, they acquired high cold-hardiness, a characteristic often observed in insects in diapause (4). Finally, and more importantly, they resumed development after being exposed to chilling conditions, which is the environmental stimulus to break diapause in many insect species (4). These developmental and survival characteristics were indistinguishable from those of the normal diapause-destined larvae. Many researches suggested that larval and pupal diapauses are maintained by ecdysteroid deficiency (37–43). The hemolymph ecdysteroid titer was kept at a low level also in diapausing H. basipunctalis larvae. However, the endocrinological cue to trigger diapause is still not very clear, whereas a low ecdysteroid titer is obviously necessary for insects to enter diapause. Our results strongly suggest that a reduction in the ecdysteroid titer is a key signal to coordinate multiple diapause-associated physiological changes in N. basipunctalis including developmental arrest, enhancement of cold hardiness, and programming to resume development, although more detailed measurements of ecdysteroid titer and examination of other physiological properties would be necessary to achieve the final goal. It would also be interesting to use E22O to examine whether ecdysteroids play a similar role in inducing larval or pupal diapause in other insects.

In conclusion, the potent ecdysteroid inactivation enzyme E22O could be used as an artificial ecdysteroid titer-reducing tool to manipulate multiple ecdysteroid-regulated phenomena. Particularly, injection of the recombinant E22O protein, a relatively simple technique, is potentially applicable to all insects and other arthropods. Judicious applications of methods utilizing E22O could obtain answers to a wide range of ecdysteroid-associated developmental and physiological questions, including uncovering new functions of ecdysteroids.

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