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Comparative study of sex pheromone composition and biosynthesis in *Helicoverpa armigera*, *H. assulta* and their hybrid

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Abstract

Two *Helicoverpa* species, *H. armigera* and *H. assulta* use (*Z*)-11-hexadecenal and (*Z*)-9-hexadecenal as their sex attractant pheromone components but in opposite ratios. Since both female and male interspecific hybrids produced by female *H. assulta* and male *H. armigera* have been obtained in our laboratory, we can make a comparative study of sex pheromone composition and biosynthesis in the two species and their hybrid. With GC and GC–MS analyses using single gland extracts, the ratio of (*Z*)-9-hexadecenal to (*Z*)-11-hexadecenal was determined as 2.1:100 in *H. armigera*, and 1739:100 in *H. assulta*. The hybrid has a ratio of 4.0: 100, which is closer to that of *H. armigera*, but significantly different from *H. armigera*. We investigated pheromone biosynthesis with labeling experiments, using various fatty acid precursors in *H. armigera*, *H. assulta* and the hybrid. In *H. armigera*, (*Z*)-11-hexadecenal is produced by Δ11 desaturation of palmitic acid, followed by reduction and terminal oxidation; (*Z*)-9-hexadecenal results from Δ11 desaturation of stearic acid, followed by one cycle of chain shortening, reduction and terminal oxidation. Δ11 desaturase is the unique desaturase for the production of the two pheromone components. In our Chinese strain of *H. assulta*, palmitic acid is used as the substrate to form both the major pheromone component, (*Z*)-9-hexadecenal and the minor one, (*Z*)-11-hexadecenal. Our data suggest that Δ9 desaturase is the major desaturase, and Δ11 desaturase is responsible for the minor component in *H. assulta*, which is consistent with previous work. However, the weak chain shortening acting on (*Z*)-9 and (*Z*)-11-octadecenoic acid, which is present in the pheromone glands, does occur in this species to produce (*Z*)-7 and (*Z*)-9-hexadecenoic acid. In the hybrid, the major pheromone component, (*Z*)-11-hexadecenal is produced by Δ11 desaturation of palmitic acid, followed by reduction and terminal oxidation. The direct fatty acid precursor of the minor component, (*Z*)-9-hexadecenoic acid is mainly produced by Δ9 desaturation of palmitic acid, but also by Δ11 desaturation of stearic acid and one cycle of chain shortening. The greater relative amounts of (*Z*)-9-hexadecenal in the hybrid are due to the fact that both palmitic and stearic acids are used as substrates, whereas only stearic acid is used as substrate in *H. armigera*. The evolutionary relationships between the desaturases in several *Helicoverpa* species are also discussed in this paper.
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1. Introduction

Sex pheromones produced by female moths are generally 12- to 18-carbon straight chain compounds, usually containing an oxygenated functional group (acetate ester, alcohols, or aldehydes) and zero to three double bonds (Tamaki et al., 1985). Moths rely on variation of sex pheromones in the chain length, the type of oxygenated functional group, and the number, location, and isomeric nature of the double bonds, as well as the precise ratios of components in multi-component pheromones to promote reproductive isolation. Closely related moth species or strains usually share common sex pheromone components but have

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different component ratios. Comparisons of pheromone communication systems of closely related species or strains have given insight into how diversification of pheromone blends may have occurred (e.g. Roelofs and Comeau, 1969; Tumlinson et al., 1974; Klun et al., 1979; Roelofs and Brown, 1982; Löfstedt et al., 1991). Further, pheromone studies involving moth hybridization as well as their pheromone biosynthesis have been extremely informative to elucidate the nature of genetic changes that have occurred (Roelofs et al., 1987; Löfstedt et al., 1989). However, information on pheromone genetics from pheromone biosynthesis in parent species and their hybrids is limited to a study of regulation of the specific ratio of the sex pheromone components in two pheromone strains of the European corn borer, *Ostrinia nubilalis*, and the intraspecific hybrid (Zhu et al., 1996). The sex pheromone of E strain of *O. nubilalis* consists of E11-14:OAc[1] with 1–3% Z11-14:OAc, whereas the sex pheromone of the Z strain of *O. nubilalis* consists of the approximately opposite blend. The female hybrids produce a 70:30 ratio between the two acetates (Klun and Maini, 1979; Roelofs et al., 1987). The *in vivo* experiments with labeled FAs as pheromone precursors suggest that the reductase system converts the E11 and Z11-14:Acid to the corresponding alcohols rather than other biosynthetic enzymes regulating the specific ratios of the two pheromone components in the two strains and their hybrid (Zhu et al., 1996).

In the genera *Helicoverpa* and *Heliothis*, almost all species investigated so far, use Z9-16:Ald and Z11-16:Ald as their sex attractant pheromone, which is necessary for eliciting male attraction to the conspecific female, with Z11-16:Ald being the major component in the ratio, except for *Helicoverpa assulta* in which Z9-16:Ald dominates (Arn et al., 2001; Cork et al., 1992). *H. armigera* and *H. assulta* are sympatric, closely related species in and around China. In *H. armigera*, the ratio of the major pheromone component, Z11-16:Ald and the minor component, Z9-16:Ald varied from 100:6.5 to 100:2.5 (Nesbitt et al., 1980; Wu et al., 1997; Dunkelblum et al., 1980; Kehat and Dunkelblum, 1990). The variation could have been caused by collection of experimental insects from different areas. An approximately opposite ratio (7:100) of the two components was

produced by females of *H. assulta* in China as their sex attractant pheromone (Liu et al., 1994) and this ratio was shown to vary with insect distribution and the pheromone release times in a light–dark cycle, but Z9-16:Ald was always a dominant component (Cork et al., 1992; Park et al., 1996). With species-specific ratios of the same sex pheromone components, these closely related species achieve reproduction isolation. A major question is how the species-specific ratio of the pheromone components is regulated. It has been shown that the desaturation of saturated FAs, one of the key steps in pheromone biosynthesis of *H. zea* and *H. assulta*, plays a major part in such regulation. In *H. assulta*, Δ9 desaturase is the major desaturase, which acts on palmitic acid to form Z9-16:Acid, the precursor of the main pheromone component Z9-16:Ald. Also Δ11 desaturase catalyses the same substrate to produce Z11-16:Acid which is then converted to the minor component, Z11-16:Ald. In *H. assulta* pheromone glands, no β-oxidation (limited chain shortening) activity was detected (Choi et al., 2002). However, the occurrence of Z7-16:Ald and Z7-16:OAc as minor components of pheromone blends from a Japanese strain of *H. assulta* suggests that residual β-oxidation of the Z9-18:Acid precursor probably occurs in this species in some geographic regions (Jeong et al., 2003). In *H. zea*, Δ11 desaturase acts on palmitic and stearic acid, the Z9-16:Acid is mainly produced by chain shortening of Z11-18:Acid, and finally the opposite ratio of the two pheromone components is produced (Choi et al., 2002).

Wang and Dong (2001) discovered that *H. armigera* could be hybridized with *H. assulta*. Only male but not female offspring was produced by the cross of female *H. armigera* and male *H. assulta*. However, the reverse cross (female *H. assulta* × male *H. armigera*) produced both fertile male and female offspring with a sex ratio of nearly 1:1. This discovery gives us a chance to make a comparative study of pheromone composition and pheromone biosynthetic pathways in both sibling species and their hybrid. In this study, in addition to pheromone composition, we report on the pheromone biosynthetic pathway in *H. armigera*, *H. assulta* and the interspecific hybrid by labeling experiments using various FA precursors. We were especially interested in determining how Δ9 and Δ11 desaturases are involved in the pheromone biosynthesis in the hybrid.

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[1]Abbreviations used: Pheromone compounds and fatty acyl moieties are abbreviated in a standard way including (in order) geometry of the double bond, position of unsaturation, chain length followed by a colon and functionality. For example, Z5-12:Ald is (Z)-5-dodecenal, Z9-16:OH is (Z)-9-hexadecenol, Z9-16:OAc is (Z)-9-hexadecenyl acetate, Z9-16:Me is methyl (Z)-9-hexadecenoate. FA = fatty acid; ME = methyl ester; FAME = fatty acid methyl ester; DMSO = dimethylsulfoxide; D_3-16:Acid = [16,16,16 $^2H_3$]-hexadecanoic acid; D_3-Z11-16:Ald = [16,16,16 $^2H_3$]-(Z)-11-hexadecenal; D_3-18:Acid- [18,18,18-$^2H_3$]-octadecanoic acid; D_4-Z11-18:Acid = [15,15,16,16-$^2H_4$]- (Z)-11-octadecenoic acid. GC = gas chromatography; GC-MS = gas chromatography- mass spectrometry; PBAN = pheromone biosynthesis activating neuropeptide.

2. Materials and methods

2.1. Insect sources and pheromone extraction

Laboratory cultures of *H. armigera* and *H. assulta* were collected in Zhengzhou, Henan province of China. The larvae were reared on an artificial diet (Wang and Dong, 2001) at $26 \pm 1\,^\circ\text{C}$ under a reversed 16:8 light:-

dark cycle. Pupae were sexed and allowed to emerge separately. A honey solution (10%) was provided for adults. The hybrids were produced in a $15 \times 15 \times 20$ cm paper container containing 7–8 pairs of female *H. assulta* and male *H. armigera*. The hybrids were reared under the same conditions as their parental species. Virgin female adults in their third scotophase were used throughout this study. For determination of pheromone components, a single pheromone gland was dissected at 5–7 h into the scotophase and extracted with 5 µl hexane containing 10 ng Z5-12:Ald.

2.2. Chemicals

Deuterium labeled FAs, D_3-16:Acid and D_3-18:Acid were purchased from Larodan Fine Chemicals, Sweden. D_4-Z11-18:Acid was a gift from Dr. R. Adlof (Northern Regional Research Centre, USDA-ARS, Peoria, IL). Reference pheromone compounds were obtained from our laboratory storage; FAMEs were prepared from the corresponding alcohols or their acetates as described by Bjostad and Roelofs (1984).

2.3. Labeling experiment

The pheromone precursors, D_3-16:Acid, D_3-18:Acid, and D_4-Z11-18:Acid, were used to monitor the biosynthetic pathway. Deuterium-labeled compounds dissolved into DMSO (8 µg/gland) were topically applied to a pheromone gland at 5–7 h into the scotophase. In some of the treatments, just after application of labeled compounds, a saline solution of the head extracts (2 head equivalents) containing endogenous PBAN was injected into the female abdomen. The glands were incubated with an applied labeled compound for 1 h at room temperature, and then 4 glands were removed and extracted with 20 µl hexane containing 40 ng Z5-12:Ald as an internal standard (four glands were used as one replicate throughout this study, unless otherwise mentioned). After removal of the hexane solution, the remaining four glands were further extracted for fatty acyl moieties with 40 µl chloroform/methanol (v/v = 2 : 1). Base methanolysis was performed to convert fatty acyl moieties to the corresponding MEs (Foster and Roelofs, 1996). Hexane containing 40 ng 13-14:Me was used for extraction of FAMEs in one replicate.

2.4. GC and GC–MS analyses

The pheromone compounds and FAMEs derived from methanolysis were identified by comparing their retention times with those of reference compounds on a Hewlett-Packard HP 5890IIGC equipped with a flame ionization detector and a capillary column (BP-20, 50 m × 0.25 mm i.d., SGE). The oven was temperature programmed 80 °C for 1 min, then 10 °C/min to 210 °C and held for 20 min. All tentatively identified compounds were confirmed by GC–MS in the scan mode using an Agilent 5973N mass selective detector coupled with an Agilent 6890N network GC system equipped with a capillary column (BP-70, 50 m × 0.25 mm i.d., SGE). The oven temperature program used in GC–MS analysis was the same to that used in GC analysis.

Incorporation of the precursors into pheromones and fatty acyl moieties was determined by using the same GC–MS instrument as used for the identification of pheromones and related FAMEs but in the selective ion monitoring mode (SIM). The following characteristic ions were used to detect unlabeled pheromone compounds or FAMEs: m/z 222 for 16:Ald, Z9-16:OAc, Z11-16:OAc, Z9-16:OH and Z11-16:OH, m/z 220 for Z7-16:Ald, Z9-16:Ald and Z11-16:Ald, m/z 270 for 16:Me, m/z 236 for Z7-16:Me, Z9-16:Me and Z11-16:Me, m/z 298 for 18:Me, and m/z 264 for Z9-18:Me and Z11-18:Me. Labeled compounds were detected using the characteristic ion + 3 when D_3-16:Acid and D_3-18:Acid were administered to the gland and the characteristic ion + 4 when D_4-Z11-18:Acid was used. The amounts of labeled and unlabeled compounds analyzed by SIM-GC-MS or FID-GC were determined using the internal standard.

3. Results

3.1. Composition of sex pheromone components and related compounds in H. armigera, H. assulta and their hybrid

In *H. armigera*, the ratio of the two pheromone components, Z9-16:Ald to Z11-16:Ald was 2.1:100, which was very close to that of the corresponding potential pheromone precursors, Z9-16:OH and Z11-16:OH (Table 1). In *H. assulta*, the ratio of Z9-16:Ald to Z11-16:Ald was 1739:100, which was also very close to that of two corresponding potential pheromone precursors, Z9-16:OH and Z11-16:OH. In addition to the above compounds, the ratio of Z9-16:OAc to Z11-16:OAc was also very close to that of the corresponding aldehydes in *H. assulta*, but the standard deviations for the two compounds were very high (Table 1). In the hybrid, Z9-16:Ald, Z11-16:Ald, Z9-16:OH, Z11-16:OH, Z11-16:OAc, and Z9-16:OAc were all detected. The ratio of Z9-16:Ald to Z11-16:Ald was close but significantly higher (*t*-test, $P \leqslant 0.01$) to that in *H. armigera*. The relative amounts of Z9-16:OH and Z11-16:OH to Z11-16:Ald were all much higher than that in *H. armigera*, but the ratio of Z9-16:OH to Z11-16:OH followed a similar pattern to that in *H. armigera*. In comparison, relatively small amounts of two new components, Z9 and Z11-16:OAc were found in the

Table 1
Relative amounts of sex pheromone components and related compounds in *H. assulta*, *H. armigera* and their hybrid

Components	Relative amount in single pheromone gland extracts (means ± SD)[a]		
	H. assulta	*H. armigera*	Hybrid
Z7–16:Ald	49.0 ± 22.6	1.2 ± 0.4	3.8 ± 1.7
Z9–16:Ald	1739 ± 390	2.1 ± 0.5	4.0 ± 1.5
Z11–16:Ald	100	100	100
Z9–16:OAc	3344 ± 4030	0	0.3 ± 0.5
Z11–16:OAc	200 ± 271	0	3.3 ± 4.6
Z9–16:OH	337 ± 156	0.1 ± 0.1	1.3 ± 1.1[b]
Z11–16:OH	26.9 ± 14.1	5.8 ± 3.1	16.7 ± 8.9[b]

[a]Means are calculated from following replicates: 17 for *H. armigera*; 11 for *H. assulta* and 16 for the hybrid.
[b]Calculated from 4 replicates.

gland extracts of the hybrid. The ratio of Z9-16:OAc to Z11-16:OAc was similar to that of the corresponding alcohols in *H. armigera* but very different from that of corresponding acetates in *H. assulta* (Table 1).

3.2. Incorporation of labeled FAs into the sex pheromone components and precursors of H. armigera

The FAs involved in the pheromone biosynthesis were determined as MEs from single gland extracts of *H. armigera*. The relative amounts of 16:Acid, Z7-16:Acid, Z9-16:Acid, Z11-16:Acid, 18:Acid and Z11-18:Acid compared to the most abundant FA, Z9-18:Acid were $58.0 \pm 9.2\%$, $4.8 \pm 1.7\%$, $10.4 \pm 2.0\%$, $7.4 \pm 2.1\%$, $25.6 \pm 4.0\%$ and $1.7 \pm 0.3\%$, respectively ($N = 13$).

In *H. armigera*, when D_3-16:Acid was used, the label was significantly incorporated into Z11-16:Ald and its precursor, Z11-16:Acid with higher incorporation, 2613 ± 1033 and 19773 ± 10508 pg/gland, respectively. However, neither labeled Z9-16:Acid nor labeled Z9-16:Ald was detected even though 20–23 glands were combined together as one replicate or after the female moths had been injected with head extracts of *H. armigera* containing PBAN activity (Table 2 and Fig. 1b). Compared with labeling results of D_3-16:Acid, when D_3-18:Acid was applied, smaller amounts of labeled Z11-16:Acid and Z11-16:Ald were produced; at the same time, small but significant amounts of D_3-Z11-18:Acid, D_3-Z9-16:Ald and trace amounts of D_3-Z9-16:Acid were also detected. A gland component, Z7-16:Ald was not labeled but its potential precursors, Z9-18:Acid was labeled by the gland incubation with D_3-18:Acid. Finally, D_4-Z11-18:Acid was incorporated into both D_4-Z9-16:Ald and D_4-Z9-16:Acid with large amounts of incorporation (Fig. 1d and Table 2).

3.3. Incorporation of FAs into the sex pheromone components and precursors of H. assulta

The FAs involved in the pheromone biosynthesis were determined as MEs from single gland extracts of *H.*

assulta. The relative amounts of 16:Acid, Z7-16:Acid, Z9-16:Acid, Z11-16:Acid, 18:Acid, and Z11-18:Acid compared to the most abundant FA, Z9-18:Acid, were $62.3 \pm 9.3\%$, $7.0 \pm 3.1\%$, $21.5 \pm 2.6\%$, $0.9 \pm 0.2\%$, $24.4 \pm 5.6\%$ and $1.7 \pm 0.3\%$, respectively ($N = 5$).

In *H. assulta*, when D_3-16:Acid was utilized, the label was significantly incorporated into Z9-16:Ald and Z11-16:Ald with much more labeled Z9-16:Ald than labeled Z11-16:Ald (Table 2 and Fig. 1b). Accordingly, both Z9-16:Acid and Z11-16:Acid were also significantly labeled by D_3-16:Acid with incorporated amounts of 5419 ± 2462 and 430 ± 298 pg/gland, respectively. Compared with *H. armigera*, much larger amounts of D_3-Z9-18:Acid as well as certain amounts of D_3-Z7-16:Acid and D_3-Z7-16:Ald were produced from the incubation with D_3-18:Acid (Table 2 and Fig. 1c). When using D_3-18:Acid with a large sample size (19–23 pheromone glands/replicate), a poorer separation interfered to quantify amounts of labeled Z11-18:Acid (data not shown) because Z9 and Z11-18:Acid were overloaded in the capillary column. However, a trace amount of D_3-Z11-18:Acid could be found after application of D_3-18:Acid on the glands of the females treated by the head extracts of *H. assulta*, but its derivatives, D_3-Z9-16:Acid and D_3-Z9-16:Ald were not found (Table 2). As D_4-Z11-18:Acid was applied, both Z9-16:Ald and Z9-16:Acid were labeled in this species, with lower incorporated amounts.

3.4. Incorporation of FAs into the sex pheromone components and precursors of the hybrid

The FAs involved in the pheromone biosynthesis were determined as MEs from single gland extracts of hybrids. The relative amounts of 16:Acid, Z7-16:Acid, Z9-16:Acid, Z11-16:Acid, 18:Acid and Z11-18:Acid compared to the most abundant FA, Z9-18:Acid were $67.8 \pm 9.3\%$, $2.8 \pm 0.6\%$, $6.3 \pm 0.8\%$, $3.7 \pm 1.0\%$, $39.9 \pm 3.5\%$ and $0.9 \pm 0.2\%$, respectively ($N = 10$).

In the hybrid, D_3-16:Acid was significantly incorporated into Z9-16:Ald and Z11-16:Ald with the ratio of

Table 2
Incorporation of various labeled FAs into the pheromone components and related compounds (fatty acid intermediates were analyzed as MEs) in the pheromone glands of *H. armigera*, *H. assulta* and their hybrid

FA applied to glands	Mean (pg/gland ± SD)							
	D_n-Z7-16:Ald	D_n-Z9-16:Ald	D_n-Z11-16:Ald	D_n-Z7-16:Me	D_n-Z9-16:Me	D_n-Z11-16:Me	D_n-Z9-18:Me	D_n-Z11-18:Me
H. armigera								
None (15)[a]	0	0	0	0	0	0	0	0
D_3-16:Acid (5)[b]	0	0	2613±1033**	0	0	19773±10508*	0	0
D_3-18:Acid (8)	0	48±21***	534±296**	0	Trace[c]	843±382**	248±71**	134±72*
D_4-Z11-18:Acid (5)	—	363±102**	0	—	3676±1012**	0	—	95525±17364
H. assulta								
None (9)	0	0	0	0	0	0	0	0
D_3-16:Acid (6)	0	443±306*	55±50*	0	5419±2462**	430±298*	0	0
D_3-18:Acid (10)[d]	20±6**	0	0	167±72**	0	0	3201±1639**	Trace[c]
D_4-Z11-18:Acid (4)	—	90±25	0	—	389±133*	0	—	33518±4239
Hybrid								
None (5)	0	0	0	0	0	0	0	0
D_3-16:Acid (6)	0	4±2**	104±57**	0	263±100**	1477±642**	0	0
D_3-18:Acid (7)	0	32±13***	300±94***	0	70±15***	340±117**	315±164*	217±134*
D_4-Z11-18:Acid (4)	—	300±79**	0	—	877±144**	0	—	29321±6651

[a]Absolute incorporations (means ± SD) were calculated from different replicates shown in parentheses. Asterisk indicates that the mean is significantly different from the control (no FA applied to gland) at various levels (*$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$; *t*-test).

[b]20–23 pheromone glands were used for one replicate.

[c]The amount was below the minimally detectable level for quantification (3 pg for aldehydes; 28 pg for FAMEs).

[d]In this experiment the pheromone production of female moths were stimulated with the head extracts, a trace amount of D_3-Z11-18:Me was detected in 8 replicates.
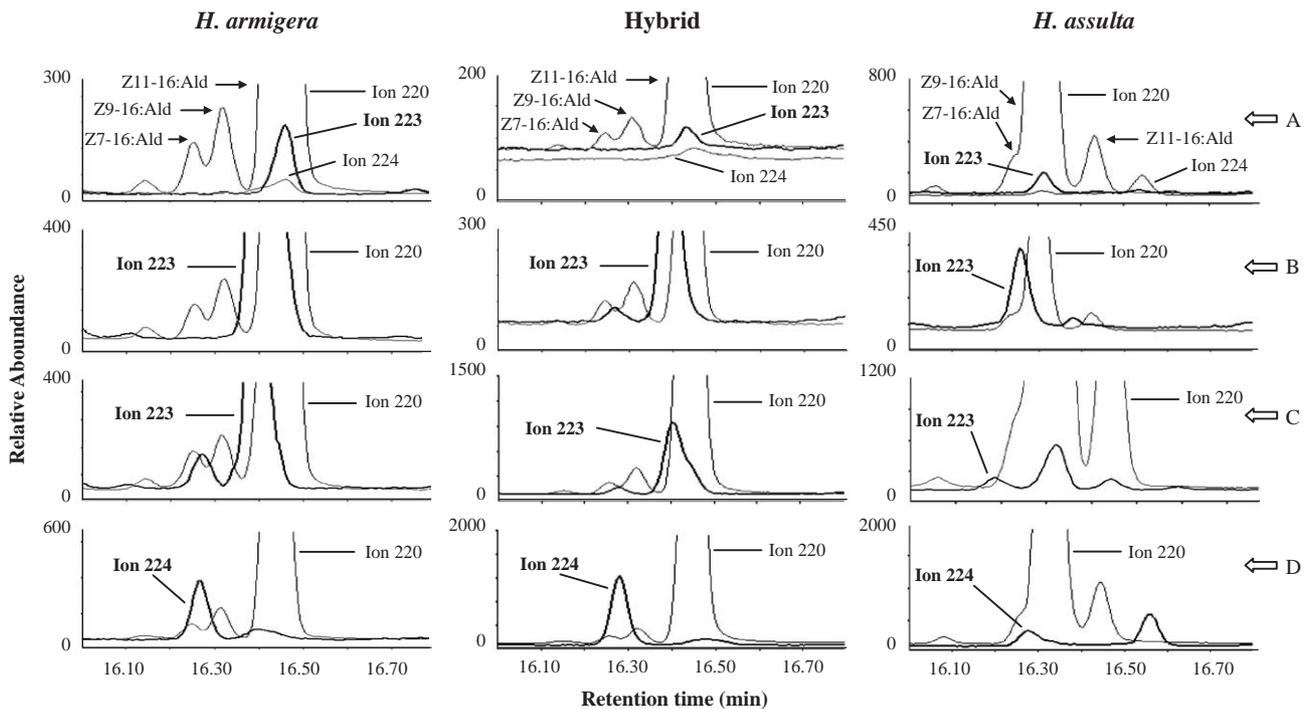

Fig. 1. Representative partial chromatograms from SIM-GC-MS analyses (BP-70 capillary column) of hexane extracts of pheromone glands after incubation with 0.4 μl DMSO (a) or 0.4 μl DMSO containing 8 μg of D_3-16:Acid (b), D_3-18:Acid (c) and D_4-Z11-18:Acid (d). The GC/MS was set in the selective ion mode and the ions 220, 223 and 224 are shown. Ion 223 and 224 represent the label incorporation from the deuterium-labeled substrates.

labeled Z9-16:Ald to labeled Z11-16:Ald being 3.5:100, which is very close to that of the corresponding natural components (Table 2 and Fig. 1b). Both Z9-16:Acid and Z11-16:Acid were also significantly labeled with a ratio of 17.8:100. The application of D$_3$-18:Acid to the pheromone glands resulted in a significant incorporation into Z9-16:Acid, Z11-16:Acid and the corresponding aldehydes compared to the control. In this case, the ratio of labeled Z9-16:Ald to labeled Z11-16:Ald (10.6:100) was higher than that produced by the application of D$_3$-16:Acid. Similar to *H. armigera*, certain amounts of D$_3$-Z11-18:Acid and D$_3$-Z9-16:Acid were produced after the application of D$_3$-18:Acid. D$_4$-Z11-18:Acid could also be significantly incorporated into the Z9-16:Ald and Z9-16:Acid in the hybrid, with much higher amounts incorporated than that in *H. assulta*.

4. Discussion

4.1. Differences in sex pheromone composition in H. armigera, H. assulta and their hybrid

The related species, *H. armigera* and *H. assulta* maintain their reproductive isolation by producing different blends of the common sex attractant pheromone components, Z9-16:Ald and Z11-16:Ald. With analyses of single pheromone gland extracts, the precise ratio of the sex pheromone components, Z9 and Z11-16:Ald was determined as 2.1:100 for a Chinese population of *H. armigera*. This ratio is very close to that found in Israel (Kehat and Dunkelblum, 1990). With the same method, we determined the ratio of the sex pheromone components, Z9 to Z11-16:Ald as 1739:100 for a Chinese strain of *H. assulta*, and this value is only slightly lower than that reported by Cork et al. (1992) and Liu et al. (1994). Relative amounts of Z11 and Z9-16:OAc found in the pheromone gland of *H. assulta* were even much higher than that of the corresponding aldehydes (Table 2). The two acetates were identified as the sex pheromone components in a Korean population (Park et al., 1996), but not in the Chinese strain (Liu et al., 1994). In the pheromone gland of the hybrid, the ratio of Z9-16:Ald to Z11-16:Ald was determined as 4.0:100. Though the amount of Z9-16:Ald is still much smaller than that in *H. assulta*, it is doubled relative to that in *H. armigera*.

The genetics of the pheromone composition of the hybrid is very similar to that of another interspecific hybrid, produced by *Heliothis virescens* and *H. subflexa* (Teal and Tumlinson, 1997). Both species use Z11-16:Ald as the major component, Z9-16:Ald as a minor one in their female sex pheromones, but the relative amount of Z9-16:Ald to Z11-16:Ald is much higher in *H. subflexa* than that in *H. virescens* (Arn et al., 2001).

Females of hybrids, either V-line (female *H. virescens* × male *H. subflexa*) or S-line (female *H. subflexa* × male *H. virescens*), produced very similar pheromone blends which resemble that of *H. virescens* but with a higher ratio of Z9-16:Ald to Z11-16:Ald.

The greater relative amounts of Z9-16:Ald and its corresponding precursor, Z9-16:OH indicated that the characteristics of the biosynthetic enzymes in the hybrid are derived from both *H. armigera* and *H. assulta*. In the hybrid, two acetate compounds, Z9-16:OAc and Z11-16:OAc were detected in the pheromone glands. These were new components compared to those found in *H. armigera*, but ratios of these components to Z11-16:Ald were much smaller than that in *H. assulta* (Table 1). Thus, the characteristics of the pheromone and related compounds in the hybrid suggest that the production of these compounds are under polygenic control as reported for the V- or S-line hybrids produced by *H. virescens* and *H. subflexa*.

4.2. Sex pheromone biosynthesis in H. armigera

In *H. armigera* D$_3$-16:Acid was only incorporated into Z11-16:Acid and the major pheromone component, Z11-16:Ald, but not into Z9-16:Acid and Z9-16:Ald although a large sample size or the females injected by PBAN-containing extracts to stimulate pheromone production were used. This suggested that Z9-16:Ald could not be produced from Δ9 desaturase acting on 16:Acid. However, D$_3$-18:Acid could be incorporated into both Z9 and Z11-16:Acid and their corresponding aldehydes. Therefore, we propose that Z9-16:Acid is produced by Δ11 desaturation of 18:Acid and one cycle of 2-carbon chain shortening, and 18:Acid can also be chain shortened to 16:Acid which subsequently produces Z11-16:Acid by Δ11 desaturation (Fig. 2). This was further proved by two other labeling experiments: one was with a mixture of D$_3$-16:Acid and D$_3$-18:Acid; another was with D$_4$-Z11-18:Acid. In the former experiment, the glands produced labeled Z11 and Z9-16:Ald with a ratio of 100:1.7 (data not shown), which was very close to the ratio of the corresponding natural pheromone components (100:2.1). In later experiments D$_4$-Z11-18:Acid could be easily converted to D$_4$-Z9-16:Acid and the corresponding aldehyde (Table 2 and Fig. 1).

In contrast to *H. armigera*, in *H. zea* it was found that Z9-16:Acid is mainly produced by Δ11 desaturation of D$_3$-18:Acid and one cycle of 2-carbon chain shortening but also by Δ9 desaturation of palmitic acid (Choi et al., 2002). Whether a Δ9 desaturase can act on 16:Acid to produce Z9-16:Acid in *H. armigera*, may eventually be elucidated by cloning the gene(s) and expressing them in a yeast system. Similar to *H. zea*, Δ9 desaturase can act on 18:Acid to produce Z9-18:Acid, which can probably convert to the gland component, Z7-16:Ald after chain
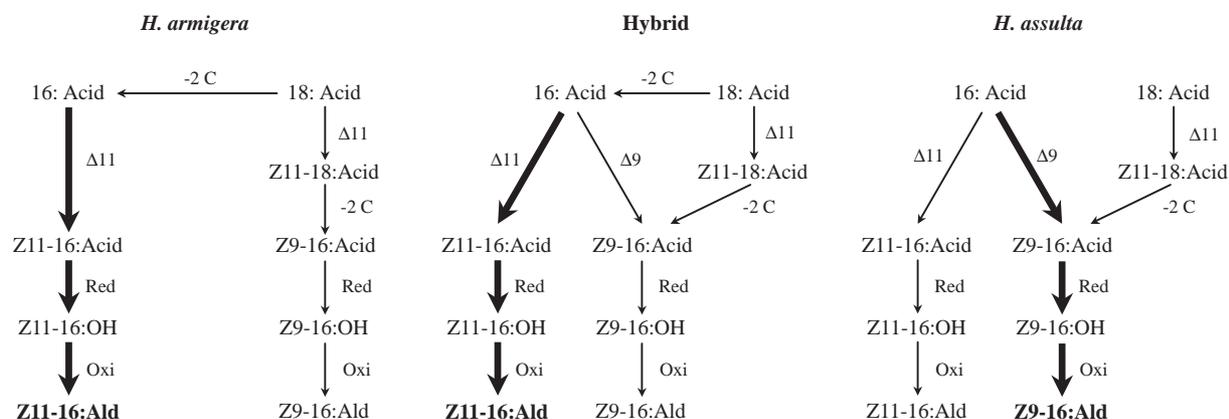
Fig. 2. Proposed biosynthetic pathways for the sex pheromone components produced by female of *H. armigera*, *H. assulta* and their hybrid. The major pathways are indicated with thick arrows. $\Delta 11$ and $\Delta 9$ means $\Delta 11$ and $\Delta 9$ desaturation, respectively; $-2C$ means one cycle of β-oxidation; Red means reduction and Oxi means terminal oxidation.

shortening, reduction and terminal oxidation. The last biosynthetic step, oxidation has been proved in many *Helicoverpa/Heliothis* species (Teal and Tumlinson, 1986; Jurenka and Roelofs, 1993) Our experiments with labeled Z9/Z11-16:Acid showed that Z9/Z11-16:Ald could be produced from the corresponding acids via the corresponding alcohols in *H. armigera* and *H. assulta* (data not shown). Thus, the biosynthetic pathway of *H. armigera* sex pheromone components, Z9/Z11-16:Ald can be determined as shown in Fig. 2.

4.3. Sex pheromone biosynthesis in H. assulta

The sex pheromone biosynthetic pathway in *H. assulta* had been identified by Choi et al. (2002). For the sake of comparing pheromone biosyntheses of two sympatric closely related species under the same experimental conditions, we studied the pheromone biosynthesis of *H. assulta* again and confirmed the results of Choi et al. (2002). $\Delta 9$ and $\Delta 11$ desaturases act on palmitic acid respectively to produce a large amount of Z9-16:Acid and a small amount of Z11-16:Acid, subsequently the two FAs can be reduced and oxidized to the major pheromone component, Z9-16:Ald and the minor one, Z11-16:Ald (Fig. 2).

However, Choi et al. (2002) found that *H. assulta* lacks chain shortening enzymes since labeled stearic acid did not label any of the 16-carbon aldehydes. Our experiments with labeled stearic acid indicated that $\Delta 9$ desaturase can act on stearic acid to produce large amounts of Z9-18:Acid, which can be chain shortened, reduced and oxidized to the gland component, Z7-16:Ald (Fig. 2 and Table 2). Another example proving the occurrence of chain-shortening is that D_4-Z11-18:Acid could also be converted to D_4-Z9-16:Acid, and then reduced to D_4-Z9-16:Ald. These results revealed that a limited chain shortening reaction does occur in the pheromone glands of *H. assulta*,

which are consistent with that found by Jeong et al. (2003). Seven desaturase cDNAs were isolated from pheromone glands of *H. assulta* and three of them were functionally expressed; these desaturase-encoding transcripts were shown to encode two $\Delta 9$ desaturases (one produces more Z9-18:Acid than Z9-16:Acid; the other produces the opposite FA profile) and one $\Delta 11$ desaturase producing only Z11-16:Acid (Jeong et al., 2003).

When comparing with *H. armigera*, much larger amounts of labeled Z9-18:Acid could be produced by $\Delta 9$ desaturase in *H. assulta*, and this leads to production of a measurable amount of labeled Z7-16:Ald. These results suggested that the relative activity or the amounts of the two $\Delta 9$ desaturases in *H. assulta* is much higher. In contrast with *H. armigera* the chain shortening reaction in *H. assulta* is much weaker. For example, the relative amount of D_4-Z9-16:Acid to Z9-16:Acid produced from D_4-Z11-18:Acid in *H. armigera* was 12 times higher than that in *H. assulta* (Table 2). In *H. assulta*, D_3-18:Acid could be also chain shortened to the corresponding 16:Acid, but the chain shortening occurred for saturated fatty acid even weaker than for unsaturated ones (data not shown). This could be a reason that both labeled pheromone components, Z9 and Z11-16:Ald were produced only from D_3-16:Acid other than D_3-18:Acid in *H. assulta*.

A trace amount of labeled Z11-18:Acid produced by $\Delta 11$ desaturation of D_3-18:Acid was detected, and D_4-Z9-16:Acid could also be produced from D_4-Z11-18:Acid. This suggested that a limited amount of Z9-16:Acid can be produced by $\Delta 11$ desaturation of stearic acid and chain shortening, while most of Z9-16:Acid is produced by $\Delta 9$ desaturation of palmitic acid (Fig. 2). Only trace amounts of labeled Z11-18:Acid and the lack of Z9-16:Acid from the experiment with D_3-18:Acid could be due to very low preference of $\Delta 11$ desaturation to 18:Acid comparing with to16:Acid.

4.4. Sex pheromone biosynthesis in the hybrid

The labeling experiments indicated that both $\Delta 9$ desaturase and $\Delta 11$ desaturase in the hybrid can act on D_3-16:Acid, which was just like the situation in *H. assulta*, but in *H. assulta* $\Delta 11$ desaturase is the more abundant or the more active desaturase compared to $\Delta 9$ desaturase. The ratio of D_3-Z9-16:Ald to D_3-Z11-16:Ald produced by the application of D_3-18:Acid was much higher than that of the corresponding natural pheromone components. This is due to the fact that D_3-Z9-16:Acid, the precursor of D_3-Z9-16:Ald could be produced by two routes: one is from $\Delta 9$ desaturation of D_3-16:Acid after chain shortening of D_3-18:Acid; another is from chain shortening of D_3-Z11-18:Acid after $\Delta 11$ desaturation of D_3-18:Acid. D_3-Z11-16:Acid was only produced by chain shortening of D_3-18:Acid and $\Delta 11$ desaturation. It appears that a combined action of $\Delta 11$ and $\Delta 9$ desaturase on 16:Acid and 18:Acid plus other downstream steps can produce both Z9-16:Ald and Z11-16:Ald with a specific ratio of 4.0:100 (Fig. 2). Unlike *H. armigera,* in which the specific ratio of Z9/Z11-16:Ald is mainly regulated by the specificity of $\Delta 11$ desaturase for different substrates, the specific ratio of Z9/Z11-16:Ald in the hybrid is mainly regulated by both $\Delta 11$ desaturase and $\Delta 9$ desaturase. In contrast to *H. assulta*, in the hybrid D_4-Z11-18:Acid could be incorporated into Z9-16:Acid and Z9-16:Ald with much larger incorporation levels (Table 2). This indicated that like *H. armigera*, chain shortening can more easily occur in the pheromone glands of the hybrid for the production of the minor pheromone component, Z9-16:Ald. In general, the pheromone biosynthesis in the hybrid is quite similar to that in *H. zea* (Choi et al., 2002).

4.5. Evolutionary relationship between the desaturases in Helicoverpa complex

In conjunction with recent studies on sex pheromone biosynthesis and acyl-CoA desaturase-encoding transcripts (Rosenfield et al., 2001; Choi et al., 2002; Jeong et al., 2003), the present study suggests that in *Helicoverpa/Heliothis* species, after the occurrence of $\Delta 11$ desaturase in *H. assulta,* the relative amounts or activity of the enzyme were increased in other related species, whereas the relative amounts or activity of $\Delta 9$ desaturase were sometimes decreased. This could result in various ratios of Z9-16:Ald to Z11-16:Ald but Z11-16:Ald is a major component in most species of the two genera. As suggested by Roelofs and Rooney (2003) the gene group encoding $\Delta 9$ desaturase producing more Z9-16:Acid than Z9-18:Acid presumably represents ancestral function in the desaturase multigene family, and during insect evolution, the first duplication event gave rise to the gene group encoding $\Delta 11$ desaturase. From our data and other recent studies, we suggest that $\Delta 9$ desaturase is the major one in *H. assulta* whereas $\Delta 11$ desaturase is the major one in *H. zea* and the unique one in *H. armigera*. From the point of view of desaturase gene evolution, it could be that *H. assulta* is the most primitive species and other two species; *H. zea* and *H. armigera* are more advanced species with *H. armigera* being further advanced. Further studies with biochemical and molecular biological approaches may provide more evidence to determine the evolutionary relationship between the desaturases in *Helicoverpa* complex.

In conclusion, the major differences between *H. armigera* and *H. assulta* in sex attractant pheromone biosynthesis are as follows: In *H. armigera*, the major pheromone component, Z11-16:Ald is produced by $\Delta 11$ desaturation of palmitic acid whereas the minor component, Z9-16:Ald only comes from $\Delta 11$ desaturation of stearic acid and subsequent chain shortening. In the Chinese population of *H. assulta*, $\Delta 9$ desaturase and $\Delta 11$ desaturase act on palmitic acid to form both the major pheromone components Z9-16:Ald and the minor one Z11-16:Ald. However, the weak chain shortening acting on Z9 and Z11-18:Acid does occur in this species and limited amounts of Z9-16:Acid can be produced by $\Delta 11$ desaturation of stearic acid and subsequent chain shortening. In contract to *H. assulta*, the sex pheromone of female hybrids produced by female *H. assulta* and male *H. armigera*, is more similar to that of female *H. armigera* in the ratio of the pheromone components, Z9/Z11-16:Ald. However, the ratio of Z9/Z11-16:Ald in the hybrid is significantly higher than that of *H. armigera*, this is due to the fact that both $\Delta 9$ desaturase and $\Delta 11$ desaturase act on palmitic and stearic acid respectively followed by chain shortening of Z11-18:Acid, thus leading to a higher ratio of the pheromone precursors Z9/Z11-16:Acid.

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