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Biotechnological potential of insect fatty acid-modifying enzymes

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Abstract: There are more than one million described insect species. This species richness is reflected in the diversity of insect metabolic processes. In particular, biosynthesis of secondary metabolites, such as defensive compounds and chemical signals, encompasses an extraordinarily wide range of chemicals that are generally unparalleled among natural products from other organisms. Insect genomes, transcriptomes and proteomes thus offer a valuable resource for discovery of novel enzymes with potential for biotechnological applications. Here, we focus on fatty acid (FA) metabolism-related enzymes, notably the fatty acyl desaturases and fatty acyl reductases involved in the biosynthesis of FA-derived pheromones. Research on insect pheromone-biosynthetic enzymes, which exhibit diverse enzymatic properties, has the potential to broaden the understanding of enzyme specificity determinants and contribute to engineering of enzymes with desired properties for biotechnological production of FA derivatives. Additionally, the application of such pheromone-biosynthetic enzymes represents an environmentally friendly and economic alternative to the chemical synthesis of pheromones that are used in insect pest management strategies.

Keywords: fatty acyl desaturases; fatty acyl reductases; lipases; pheromones.

1 Introduction

The fatty acids (FAs) and FA derivatives are a diverse group of compounds with a range of applications. They may be used as food supplements, cosmetics, adhesives, industrial lubricants, polymer plasticizers and stabilizers [1], and raw materials for further chemical processing (reviewed by Metzger and Bornscheuer [2]). Economic production of these compounds from affordable raw materials, such as hydrocarbons from petroleum refining and vegetable or animal oils, is well established at an industrial scale [3]. However, traditional procedures are not applicable to the production of unconventional FAs and FA derivatives, e.g. with double bonds in unusual positions, that might be useful for specific applications [2]. The utilization of metabolically engineered organisms or heterologous production of engineered enzymatic catalysts is promising tools for the production of such FA derivatives. Plants and microorganisms metabolically engineered to produce polyunsaturated FAs (PUFAs) [4–6] and fatty alcohols [7] have been tested. Here, we focus on the potential application of insect enzymes to the biotechnological production of unusual FA derivatives.

Many insect species use a diverse range of FA-modifying enzymes to synthesize pheromones that mediate communication among individuals of the same species. The FA-derived pheromones are the most common [8], encompassing thousands of compounds and mixtures of compounds [9]. Insect pheromone-biosynthetic enzymes presumably evolved via a divergence of the original functions of the FA-biosynthetic and FA-modifying enzymes participating in insect primary metabolism [10]. The diversification of the pheromone-biosynthetic enzymes likely has been driven by evolutionarily imposed requirements on sex pheromone signal specificity [11, 12]. A broad spectrum of insect pheromone-biosynthetic enzymes has already been functionally characterized, and transcriptomic sequencing (RNA-seq) of pheromone glands using next-generation sequencing has identified new candidates for characterization [13–19]. RNA-seq of other insect glands and tissues may open the door to the discovery of many additional enzymes with remarkable biosynthetic capabilities [8].

Among insects, moths (Lepidoptera) have received the greatest scientific attention focused on pheromone biosynthesis. The moth female sex pheromones, which attract conspecific males, are generally FA-derived alcohols, acetates, aldehydes, esters, hydrocarbons, or epoxides of various hydrocarbon chain lengths and contain zero, one, or multiple double bonds (or triple bonds) placed apart from each other, methylene interrupted, or

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conjugated. The number, position, and stereochemistry of double bond(s) in the chains of the FAs influence their biophysical properties and the properties of the resulting lipids [20] and are critical for the biological activity of the FA-derived pheromones.

Moths include numerous pest species that cause billions of Euros of damage annually in forestry and agriculture. For example, the diamondback moth (*Plutella xylostella*), a pest of rapeseed (*Brassica napus*), is estimated to cost almost 4 billion Euros annually on combined pest management and residual losses in crop production [21]. Pest management strategies that employ insect pheromones to trap and kill, monitor insect abundance, or confuse insect pheromone communication channels are environmentally friendly alternatives to traditional, widespread strategies of fighting insect pests, such as the use of insecticides. Synthetic pheromones have been used to fight pest moth species [22]; however, new biotechnological approaches in which pheromones are produced with the help of genetically modified plants or microorganisms are currently being developed [23–25]. Here, we not only review this application of insect pheromone-biosynthetic enzymes but also propose as-yet unexplored or unexploited applications that may benefit from the catalytic potential of these enzymes.

2 Insect enzymes catalyzing biosynthesis of FA-derived pheromones

Efforts to understand the basis of insect pheromone biosynthesis have generated insights into the enzymatic specificities of several classes of FA-modifying enzymes. The majority of information is available for one insect group – the moths [18]. FA-modifying enzymes from other insect orders, such as beetles (Coleoptera) [26], flies (Diptera) [27–29], crickets (Orthoptera) [30, 31] and bees (Hymenoptera) [32–34], are substantially less well explored and usually connected to primary FA metabolism rather than pheromone biosynthesis.

Two enzyme groups that are encoded by large multigene families in insects – the membrane fatty acyl desaturases (mFADs) [35, 36] and the fatty acyl reductases (FARs) [37] – have attracted the most research attention.

2.1 mFADs

The mFADs (EC 1.14.19.–) belong to a superfamily of oxygen-dependent membrane di-iron-containing enzymes that share common features including a conserved tripartite histidine-rich motif coordinating two iron ions in the active center. These enzymes catalyze the highly energy-demanding removal of hydrogen from an unactivated fatty acyl at a precise position along the hydrocarbon chain. The process involves a reactive oxo-intermediate formed by the activation of molecular oxygen. The net result of the desaturation reaction is the introduction of a double bond into the fatty acyl chain (and reduction of molecular oxygen to water) [38] (Figure 1). The evolutionarily unrelated class of apparently convergent soluble FADs [39] is expressed exclusively in the stroma of plant plastids, and these enzymes desaturate fatty acyls bound to an acyl carrier protein.

The mFADs are present in the cell membranes of some bacteria [40, 41], thylakoid and cytoplasmic membranes of cyanobacteria [42], thylakoid and cytoplasmic membranes of plants, and ubiquitously in eukaryotic endoplasmic reticulum (ER) membranes [35]. The ER mFADs, on which we focus throughout this review, use an electron pair supplied by nicotinamide adenine dinucleotide (NADH) via an electron transport system consisting of NADH:cytochrome b_5 reductase and cytochrome b_5 [38]. A slight modification of this electron-supply chain is seen in some mFADs, in which a cytochrome b_5 domain is fused to the *N*- or *C*-terminus of the desaturase [43, 44].

The research conducted on mFADs primarily aims to identify the determinants of desaturase specificity, enabling engineering of mFADs that produce economically or industrially relevant FAs, such as the PUFAs that serve as nutritional supplements or starting materials in the chemical industry [45, 46]. Another major research goal is to uncover the mechanistic details of FA desaturation, which might enable rational design of specific inhibitors targeting either the mFADs involved in human metabolic diseases, such as diabetes or obesity [47], or the mFADs essential for pathogenic microorganisms, such as pathogenic yeasts [48–50] and trypanosomatids [51, 52]. Basic research also aims to uncover the molecular basis of

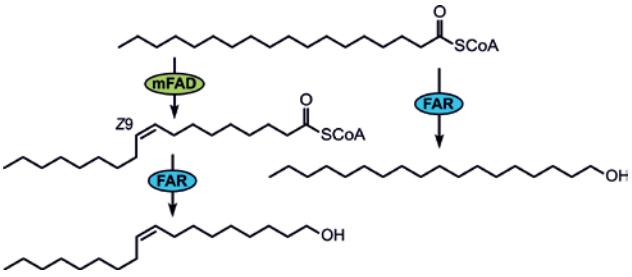

Figure 1: Schematic depiction of the reactions catalyzed by membrane fatty acyl desaturases (mFADs) and fatty acyl reductases (FARs).

insect pheromone evolution by studying the pheromone-biosynthetic mFADs [18].

2.1.1 mFAD properties

The mFADs display several enzymatic activities. They may be (i) stereospecific, i.e. introduce a double bond into fatty acyl chains in either an E or Z configuration, or (ii) regiospecific, i.e. they prefer a particular position along the fatty acyl hydrocarbon chain for double bond introduction, usually marked as ΔX or ZX-, EX- [53]. The mFADs also display diverse substrate specificities; they may prefer a particular chain length, the presence of pre-existing double bond(s) at particular position(s), stereochemical configuration and a head group of fatty acyl substrate (e.g. acyl-CoA or acyl-lipid substrates) [54]. Moreover, hydroxylated (and less commonly acetylenated, i.e. bearing a triple bond) products may accompany the desaturated products as a result of the mechanistic similarity between the reaction mechanisms [55, 56]. Several mFADs exhibit a fatty acyl conjugase activity – the ability to produce a system of conjugated double bonds by a reaction mechanism involving a shift in the position of a pre-existing double bond [57, 58]. The knowledge gained from mFAD characterization indicates that although numerous mFADs are highly specific and produce only a limited set of unsaturated products, they can follow more than one specificity mode under particular conditions, such as the presence of different substrates or during sequential biosynthesis of FAs with multiple double bonds [59, 60]. Most likely, the insect mFADs evolved an excessively wide range of specificities in connection with pheromone biosynthesis [35].

The mFADs producing *Z*9-monounsaturated FAs are the most widespread eukaryotic desaturases, followed by mFADs with *Z*5-, *Z*6-, *Z*12- and *Z*15-specificities. The majority of animal and insect mFADs desaturate fatty acyl-CoA groups, except some animal mFADs involved in PUFA biosynthesis that prefer fatty acyl lipids [61, 62]. The experimental evidence on the identity of the mFAD substrate head group is, however, scarce [54].

The first functionally characterized insect mFAD was isolated from the cabbage looper moth (*Trichoplusia ni*) and exhibited *Z*11-desaturase specificity. An FA-derived pheromone component containing a double bond at position Δ11 is present in many moth species [63]. Subsequently, more than 50 distinct insect FAD genes have been identified, cloned and functionally characterized [18, 36] (Table 1).

The moth mFADs reflect the diverse desaturase specificities in insects. In addition to the ability to introduce *Z*-double bonds, moth mFADs can catalyze the introduction of rather uncommon *E*-double bond in nature [64–68] or produce a mixture of E- and Z-unsaturated FAs [69, 70]. The preferred fatty acyl chain length may be C14 [27, 66–68, 70–73], C16 [74], or C18 [63, 75, 76], but some mFADs can desaturate a broad range of fatty acyl chain lengths, such as C14-C20 [67, 77]. The positions of the introduced double bond include Δ9, Δ4 [33], Δ5 [72], Δ6 [64, 78], Δ8 [65], Δ10 [69, 78], Δ11 [10, 63, 65–67, 74–76, 79, 80], Δ13 [81], and Δ14 [70, 82]. Δ12 mFADs have also been identified, but they are involved in primary metabolism during the production of methylene-interrupted PUFAs [31] rather than pheromone biosynthesis. The moth mFADs can also introduce a double bond at the terminal position between the penultimate and ultimate carbon atoms [78, 83] and can produce FAs with a system of isolated double bonds [64] or a system of conjugated double bonds [10, 65, 66, 76, 79, 80, 82]. Among the more bizarre desaturation reactions, an mFAD identified in the processionary moth, *Thaumetopoea pityocampa*, can introduce triple bonds into the FA chains [81], resembling in activity an mFAD described in the plant of *Crepis* genus [84]. Two moth Δ11-mFADs have been shown to exhibit minor Δ11-hydroxylation activity [85]. Table 1 shows functionally characterized insect mFADS.

2.2 FARs

The alcohol-forming FARs [EC 1.2.1.84, systematically long-chain acyl-CoA: nicotinamide adenine dinucleotide phosphate (NADPH) reductases] belong to a family of oxidoreductases and catalyze reduction of activated FAs to the corresponding fatty alcohols by means of a four-electron process employing a reduced dinucleotide (either NADPH or NADH) as a reductant [91]. The reaction takes place on the thioester moiety through a putative aldehyde intermediate, which is usually not released from the enzyme-substrate complex [92]. In addition to the alcohol-forming FARs, there are also reductase enzymes that produce aldehydes from fatty acyl-CoAs (aldehyde-forming reductases) and fatty alcohols from aldehydes (aldehyde reductases) [91, 93]. The FARs are most probably localized to ER membranes [94, 95].

The fatty alcohols, which are usually defined as primary alcohols having more than 12 carbon atoms in the chain, are naturally abundant FA derivatives that play a variety of biological roles. The fatty alcohols are precursors of waxes that serve as surface-protective compounds in plant pollen [96], preventing excessive water loss in insects [29], and are secreted as skin-, eye-, or feather-protective compounds in mammals and birds [97, 98]. The fatty alcohols are also components of ether lipids,

Table 1: Overview of functionally characterized insect fatty acyl desaturases (mFADs).

Order	Source organism	Expression system	mFAD name	Major unsaturated fatty acyl product(s)	Other unsaturated fatty acyl product(s)	Reference
Lepidoptera	*Antheraea pernyi*	*S. c.*	Ape-PGΔ11	Z11-16:1; Z11-18:1	Z/E11-14:1; E6,**Z11**-16:2	[64]
	A. pernyi	*S. c.*	Ape-PGΔ6	Z/E6-16:1	**E6**,Z11-16:2	[64]
	Argyrotaenia velutinana	*S. c.*	RBLRG-Z/E 11	Z/E11-14:1		[71]
	A. velutinana	*S. c.*	RBLRFB-Z9	Z9-16:1; Z9-18:1	Z9-14:1; Z9-15:1; Z9-17:1	[71]
	Bicyclus anynana	*S. c.*	Ban-Δ11	Z11-16:1		[86]
	Bombyx mori	Sf9	Desat1	Z11-16:1; **E10,E/Z12**-16:2	Δ9,**Δ11**-16:2	[79]
	Choristoneura parallela	*S. c.*	CpaZ9(16>18)	Z9-16:1; Z9-18:1		[67]
	C. parallela	*S. c.*	CpaZ9a(16>18)	Z9-16:1; Z9-18:1		[67]
	C. parallela	*S. c.*	CpaZ9(18>16)	Z9-18:1; Z9-16:1		[67]
	C. parallela	*S. c.*	CpaZ9(14–26)	Z9-16:1; Z9-18:1; Z9-14:1, Z9-20:1; Z9-24:1	Z9-15:1; Z9-17:1; Z9-22:1; Z9-26:1	[67]
	C. parallela	*S. c.*	CpaE11	E11-14:1		[67]
	Choristoneura rosacea	*S. c.*	Cro-Z9(16)	Z9-16:1; Z9-18:1		[73]
	C. rosacea	*S. c.*	Cro-Z/E11	Z/E11-14:1		[73]
	Ctenopseustis herana	*S. c.*	Desat7	Z5-14:1		[72]
	C. herana	*S. c.*	Desat4	Z6-14:1; Z6-16:1		[78]
	Ctenopseustis obliquana	*S. c.*	Desat7	Z5-14:1		[72]
	C. obliquana	*S. c.*	Desat6	Z9-18:1; Z9-16:1	Z9-14:1; Z9-17:1	[78]
	Dendrolimus punctatus	*S. c.*	Dpu-Δ11-APSQ	Δ8-12:1; Δ8-14:1; Δ8-16:1	Z/E9, **Z/E11**-16:2	[65]
	D. punctatus	*S. c.*	Dpu-Δ11-LPAE	Z11-16:1; Z11-18:1	Δ11-12:1; Δ11-14:1; E9,**Z/E11**-16:2	[65]
	D. punctatus	*S. c.*	Dpu-Δ9-KPSE	Z/E9-12:1; Z/E9-14:1; Z/E9-16:1; Z9-18:1	Z7, **E9**-14:2; E7, **Z/E9**-14:2	[65]
	Epiphyas postvittana	*S. c.*	LBAM-FBZ9	Z9-16:1; Z9-18:1	Z9-14:1; Z9-15:1; Z9-17:1	[66]
	E. postvittana	*S. c.*	LBAM-PGZ9	Z9-18:1; Z9-16:1	Z9-14:1; Z9-15:1; Z9-17:1	[66]
	E. postvittana	Sf9	LABM- PGE11	E11-14:1; E11-16:1, E9,**E11**-14		[66]
	Helicoverpa assulta	*S. c.*	HassKPSE	Z9-18:1; Z9-16:1		[74]
	H. assulta	*S. c.*	HassNPVE	Z9-16:1; Z9-18:1		[74]
	H. assulta	*S. c.*	HassLPAQ	Z11-16:1		[74]
	Helicoverpa zea	*S. c.*	HzPGDs1	Z11-16:1		[87]
	H. zea	*S. c.*	HzPGDs2	Z9-16:1; Z9-18:1		[87]
	Lampronia capitella	*S. c.*	Lca-QPAQ	Z9-16:1; Z9-18:1	Z9-14:1	[10]
	L. capitella	*S. c.*	Lca-KVPQ	Z11-16:1; Z11-18:1	Z11-14:1; Z11-20:1; Z9,Δ11-16:2	[10]
	Mamestra brassicae	*S. c.*	MbraLPAQ	Z11-16:1; Z11-18:1		[75]
	Manduca sexta	*S. c.*	MsexAPTQ/MsexD2	Z11-16:1	E11-16:1; Z11-18:1; E10,E12-16:2; E10,Z12-16:2	[80, 82]
	M. sexta	*S. c.*	MsexD3	Z11-16:1; E10,E12,**E14**-16:3; E10,E12,**Z14**-16:3		[82]
	M. sexta	*S. c.*	MsexD6	Z11-18:1		[82]

Table 1 (continued)

Order	Source organism	Expression system	mFAD name	Major unsaturated fatty acyl product(s)	Other unsaturated fatty acyl product(s)	Reference
	M. sexta	*S. c.*	MsexD5	E10,E12,E14-16:3; E10,E12,Z14-16:3		[82]
	Operopthera brumata	*S. c.*	Obr-LPAQ	Z11,Z14,Z17,**Z19**-20:4		[83]
	Ostrinia furnacalis	*S. c.*	Ofu9[16]	Z9-16:1; Z9-18:1		[70]
	O. furnacalis	Sf9	Ofu-Z/E14	Z/E14-16:1		[70]
	O. furnacalis	*S. c.*	Ofu-Z/E11	Z11-16:1	Z/E11-14:1	[70]
	O. furnacalis	*S. c.*	Ofu9[18]	Z9-18:1; Z9-16:1		[70]
	Ostrinia latipennis	Sf9	LATPG1	E11-14:1		[68]
	Ostrinia nubilalis	*S. c.*	Onu9[16]	Z9-16:1; Z9-18:1		[70]
	O. nubilalis	Sf9	Onu-Z/E14	Z/E14-16:1		[70]
	O. nubilalis	*S. c.*	Ofu9[18]	Z9-18:1; Z9-16:1		[70]
	O. nubilalis	*S. c.*	Onu-Z/E11	Z11-16:1	Z/E11-14:1	[70]
	Planotortrix excessana	*S. c.*	Desat3	Δ13-14:1, Δ15-16:1, Δ17-18:1		[78]
	Planotortrix octo	*S. c.*	Pocto-Z9	Z9-16:1; Z9-18:1;	Z9-14:1; Z9-15:1; Z9-17:1	[69]
	P. octo	*S. c.*	Pocto-Z10	Z10-16:1		[69]
	Spodoptera littoralis	*S. c.*	SlsZ9(16)	Z9-16:1; Z9-18:1	Z9-14:1; **Z9**,E11–14:1	[76]
	S. littoralis	*S. c.*	SlsZ9(18)	Z9-18:1; Z9-16:1	Z9-14:1; **Z9**,E11–14:1	[76]
	S. littoralis	*S. c.*	Sls-FL3	Z11-16:1; Z11-18:1	Z/E11-14:1; E10,Z12-14:2; E10,Z12-16:2; 11OH-16:0	[76, 85]
	Thaumetopoea pityocampa	*S. c.*	Tpi-PGFAD	Z11-16:1; 11-hexadecynoic acid; Z13-hexadecen-11-ynoic acid	Z/E11-14:1	[81]
	Trichoplusia ni	*S. c.*	T.niΔ9	Z9-18:1; Z9-16:1	Z9-14:1; Z9-15:1	[88]
	T. ni	*S. c.*	PDesat-TnΔ[11]Z	Z11-16:1; Z11-18:1	11OH-16:0; 11OH-18:0	[63, 85]
	Yponomeuta padellus	*S. c.*	Ypa-Δ11-desaturase	Z11-16:1; Z11-18:1; Z11-20:1	Z11-22:1; Z/E11-14:1	[89]
Orthoptera	*Acheta domesticus*	*S. c.*	Cricdes3	Z9-18:1; Z9-16:1		[30]
	A. domesticus	*S. c.*	AdD12DES	Z9,**Z12**-18:2; Z9,**Z12**-16:2		[31]
Diptera	*Drosophila melanogaster*	*S. c.*	Tai desat1	Δ9-16:1; Δ9-18:1	Δ9-14:1	[27]
	D. melanogaster	*S. c.*	Cs desat1	Δ9-16:1; Δ9-18:1	Δ9-14:1	[27]
	D. melanogaster	*S. c.*	Tai desat2	Δ9-14:1	Δ9-16:1; Δ9-18:1	[27]
	Musca domestica	*S. c.*	MdomΔ9	Δ9-16:1; Δ9-18:1		[28]
Coleoptera	*Tribolium castaneum*	*S. c.*	TcasZ9desA	Z9-18:1; Z9-16:1		[26]
	T. castaneum	*S. c.*	TcasZ9desB	Z9-18:1; Z9-16:1		[26]
	T. castaneum	*S. c.*	TcasZ12	Z9,**Z12**-18:2; Z9,**Z12**-16:2		[31]
	T. castaneum	*S. c.*	TcasD1	Δ9-12:1; Δ9-14:1; Δ9-16:1		[90]
	T. castaneum	*S. c.*	TcasD2	Δ5-16:1; Δ5-18:1		[90]
	T. castaneum	*S. c.*	TcasD3	Δ5-16:1; Δ5-18:1		[90]
	T. castaneum	*S. c.*	TcasD4	Δ9-16:1		[90]

Table 1 (continued)

Order	Source organism	Expression system	mFAD name	Major unsaturated fatty acyl product(s)	Other unsaturated fatty acyl product(s)	Reference
	T. castaneum	*S. c.*	TcasD5	Z9-18:1; Z9-16:1		[90]
Hymenoptera	*Bombus terrestris, Bombus lucorum, Bombus lapidarius*	*S. c.*	Δ9-Bter, Δ9-Bluc, Δ9-Blap	Z9-18:1; Z9-16:1, Z9-14:1		[33]
	B. terrestris, B. lucorum, B. lapidarius	*S. c.*	Δ4-Bluc/Bter, Δ4-Blap	E/Z4-14:1		[33]

In UFA nomenclature, E/ZX indicates the position of E- or Z-double bond between X and X+1 atom of fatty acyl chain; ΔX indicates position of double bond with unspecified configuration; X:Y indicates a fatty acyl chain with X carbon atoms containing Y double bonds. Mixture of both double bond configuration isomers is marked as E/Z. When unsaturated FAs served as a substrate, the double bond introduced by heterologously expressed insect FAD is highlighted in bold. 11OH-16:0 and 11OH-18:0 denote FA hydroxyderivatives. *S. c.*, *Saccharomyces cerevisiae*; and Sf9, *Spodoptera frugiperda* cell line.

which are abundant in the cell membranes of cardiac, nervous and lymphoid tissues [99]. In some plants such as jojoba (*Simmondsia chinensis*), marine crustaceans (e.g. *Calanus finmarchius*), and protists (e.g. *Euglena gracilis*), fatty alcohols serve as precursors of energy-storing waxes [100–102]. Notably, the higher volatility of fatty alcohols and acetates, as compared to the respective FAs, may have led to their utilization as airborne signals – pheromones and scents. These compounds may serve as pheromone components in reptiles [103] and some mammals, such as deer [104], but above all, they are among the main pheromone components in insects, including moths [105], termites [106], bees [107–109], and wasps (reviewed by Tillman et al. [110]).

2.2.1 FAR properties

Virtually all organisms produce one or multiple types of fatty alcohols of various chain lengths and degrees of unsaturation. The majority of the findings available to date suggest that the FARs in general exhibit diverse substrate specificities with respect to the fatty acyl chain length and unsaturation state of the substrate. The pool of fatty acyls available for reduction in the host organism can also influence the apparent FAR specificity [111–113].

The first FARs studied by genetic methods were plant FARs from jojoba and wheat [100, 114]. Heterologous expression of plant FARs in *Escherichia coli*, rapeseed (*B. napus*), and *Saccharomyces cerevisiae* led to a range of saturated and unsaturated alcohols of C14 to C26 chain length, depending on the host organism [100, 114, 115]. Besides plant FARs, microbial FAR genes [102, 116, 117] and FARs from vertebrates have also been isolated and functionally described [97, 98].

The insect reductases first received attention not long after the enzymatic characterization of their plant orthologs. They have been studied extensively, primarily in moths. In silk moth (*Bombyx mori*), researchers discovered a pheromone gland-specific FAR [118] that is able to convert *E*10,*Z*12-16:2 FA precursor to the corresponding alcohol bombykol, the main component of the female sex pheromone. Since then, a range of moth pheromone-biosynthetic FARs have been isolated and functionally characterized [89, 111, 119–123] (Table 2). Although moth FARs usually exhibit a broad substrate preference [77, 89, 111, 120, 122, 123], some of them display specificity to unsaturated substrates with a double bond in either the *E* or *Z* configuration [119], a particular chain length and double bond position [120], or a system of conjugated double bonds [118]. Given the multiple FAR paralogs generally

Table 2: Overview of functionally characterized insect fatty acyl reductases (FARs).

Order	Source organism	Expression system	FAR name	Major fatty alcohol product(s)	Reference
Lepidotera	*Agrotis segetum*	*S. c.*	AseFAR	Z9-14:1; 16:0; 14:0; Z11-16:1	[23]
	Bicyclus anynana	*S. c.*	Ban-wFAR1	16:0	[86]
	B. anynana	*S. c.*	Ban-wFAR2	Z9-14:1; 14:0	[86]
	Bombyx mori	*S. c.*	pgFAR	E/Z11-16:1; E10,Z12-16:2	[118]
	Helicoverpa armigera, H. assulta	*S. c.*	HarFAR, HasFAR	Z9-14:1; Z11-16:1; Z9-16:1; 16:0	[123]
	Heliothis virescens, Heliothis subflexa	*S. c.*	HvFAR, HsFAR	Z9-14:1; Z11-16:1; Z9-16:1; 16:0	[123]
	O. nubilalis	*S. c.*	pgFAR-Z	Z11-14:1; E12-14:1	[119, 120]
	O. nubilalis	*S. c.*	pgFAR-E	E11-14:1	[119, 120]
	Ostrinia palustralis, O. latipennis	*S. c.*	pgFAR	E11-14:1	[120]
	Ostrinia scapulalis	Sf9	FAR-XIII	E11-14:1	[111]
	Ostrinia zaguliaevi, O. furnacalis	*S. c.*	pgFAR	14:0; Z11-14:1; E/Z12-14:1	[120]
	Ostrinia zealis	*S. c.*	pgFAR	14:0; E11-14:1; E12-14:1	[120]
	Spodoptera exigua	*S. c.*	SexpgFAR I	16:0; Z11-16:1; E14-16:1; 14:0	[111]
	S. exigua	*S. c.*	SexpgFAR II	14:0; Z9-14:1; Z9,E12-14:2; E/Z11-14:1; E/Z12-14:1	[111]
	S. littoralis	*S. c.*	SlitpgFAR I	16:0; Z11-16:1; E14-16:1; 14:0	[111]
	S. littoralis	*S. c.*	SlitpgFAR II/SlitFAR1	14:0; Z9-14:1; Z9,E12-14:2; E/Z11-14:1; E/Z12-14:1	[111, 122]
	Yponomeuta evonymellus, Y. padellus, Yponomeuta rorellus	*S. c.*	pgFAR	14:0; E/Z11E-14:1; Z9-14:1; 16:0	[89]
Diptera	*D. melanogaster*	*S. c.*	Waterproof	24:0; 26:0	[29]
Hymenoptera	*Apis mellifera*	*S. c.*	AmFAR1	18:0; 16:0; 20:0; 22:0	[34]

In FA alcohol nomenclature, E/ZX indicates the position of E- or Z-double bond between X and X+1 atom of fatty acyl chain; X:Y indicates a fatty acyl chain with X carbon atoms containing Y double bonds. Mixture of both double bond configuration isomers is marked as E/Z. *S. c.*, *Saccharomyces cerevisiae*; and Sf9, *Spodoptera frugiperda* cell line.

present in insect genomes [13, 14, 16, 17, 111, 124] and limited knowledge of their properties, there is a demand to perform functional characterization of these biologically and biotechnologically relevant enzymes.

FARs from insects other than moths are virtually unexplored. A FAR presumably involved in biosynthesis of fatty alcohol precursors of waxes has been identified in *Drosophila* [29], and honeybee (*Apis mellifera*) FAR capable of reducing hydroxylated fatty acyl precursors and saturated C16-C22 fatty acyls has been characterized [34]. FARs from pheromone-producing glands in the butterfly *Bicyclus anynana* reduce C14 and C16 fatty acyls [86]. At least 13 FAR genes have been identified in the labial gland of *Bombus terrestris* by RNA sequencing [14], and these are currently being functionally characterized in our laboratory. Such a spectrum of FARs from various organisms with distinct substrate specificities presents a potential enzymatic toolbox for tailored biotechnological production of fatty alcohols. Additionally, comparative analysis of these FARs might help uncover the determinants of FAR specificity.

2.3 Other enzymes

In addition to mFADs and FARs, other FA-modifying and FA-biosynthetic enzymes have been studied both in vivo and in vitro. These include (i) acyltransferase involved in synthesis of FA-storing triacylglycerols [125, 126]; (ii) lipases and esterases involved in hydrolytic release of FA pheromone precursors from storage triacylglycerols [127–129], formation of FA ethyl esters [130], or primary metabolism [131–133]; and (iii) cytochrome P450, involved in the oxidative decarbonylation of FAs to hydrocarbons [134]. However, for the majority of insect pheromone biosynthetic steps, the genes and enzymes involved have yet to be identified and characterized. Among these are enzymes catalyzing (i) FA elongation [135, 136] and FA chain shortening [137–140]; (ii) epoxide group formation [141, 142]; (iii) acetate ester formation [143, 144]; (iv) oxidation of fatty alcohols to aldehydes [19, 145–147]; and (v) FA biosynthesis, e.g. acetyl-CoA carboxylase [148] and fatty acid synthase [149].

3 Enzyme engineering

The specificity of insect pheromone-biosynthetic enzymes can evolve abruptly, presumably as a consequence of their role in reproductive isolation and speciation [11, 12]. The process of functional divergence of pheromone-biosynthetic enzymes, which can be observed in insect subpopulations or closely related species, generates biocatalysts that are highly similar in protein sequence yet distinct in their enzymatic properties. These enzymes are convenient model systems to study the mechanisms of enzyme specificity determinants [82, 119, 150]. Importantly, the amassed knowledge about the function of the FA-modifying enzymes could be used to design novel enzymes with desired enzymatic properties.

3.1 Structural determinants of mFAD function

Substantial research effort has been put into the identification of specificity determinants of mFADs by either random mutagenesis or rational mutagenesis guided by topology predictions and sequence comparisons of mFADs with distinct specificities. The mFADs and their mutants are typically functionally characterized in the yeast *S. cerevisiae* [151] or in baculovirus-insect cell expression systems [79]. These experiments led to the identification of sequence determinants of both acyl-CoA and acyl-lipid mFAD specificities in diverse organisms, such as transmembrane helices or conserved histidine-rich motifs [55, 59, 152–162]. Buček et al. [82] identified a critical amino acid residue in the transmembrane domain of an mFAD from *Manduca sexta* (*Msex*D3) that determines the specificity and ability of this desaturase to catalyze biosynthesis of FAs containing three conjugated double bonds via *E*/*Z*14 desaturation from diunsaturated FAs.

Recently, the crystal structures of two closely related mammalian mFADs with bound fatty acyl-CoA substrate provided the first direct structural insights into the mFADs [163, 164]. In agreement with previous topology predictions [151] and topology-mapping experiments [165], the crystal structures revealed four transmembrane α-helices and a large extramembrane portion of the enzyme including the active center localized on the cytosolic side of the ER membrane. The di-iron active center is coordinated by an ordered water molecule and nine conserved histidine residues. Eight of the coordinating histidines are organized in a tripartite histidine-rich motif, which was previously shown to be essential for mFAD activity [166]. The crystal structures provided direct experimental evidence for a kinked narrow hydrophobic substrate-binding tunnel, which extends approximately 24 Å into the enzyme interior and binds the fatty acyl tail of the fatty acyl-CoA substrate [164]. The kink in the binding tunnel is hypothesized to play a role in correct positioning of the fatty acyl chain toward the active center.

The availability of an mFAD crystal structure enables homology modeling of other related mFADs and can help increase understanding of experimentally obtained biochemical data. Indeed, the homology model of *Msex*D3 highlighted the prominent position of the critical residue Ile224 in the kink of the fatty acyl substrate binding tunnel, which presumably plays a critical role in positioning the substrate fatty acyl chain with respect to the di-iron active center [82]. Ding et al. found that reciprocal exchange of a single amino acid residue in moth Δ11 mFADs can switch between their E and Z desaturase specificities. The critical residue 258Glu/Asp is predicted to form a secondary coordination sphere of the active center iron ions, based on homology models [150].

3.2 Structural determinants of FAR function

The information about FAR structures is very limited. The *N*-terminal motif (IVF)X(ILV)TGXTGFL(GA) belonging to the Rossmann fold $NAD(P)^+$ binding domain and the *C*-terminal FAR_C domain is conserved among the FARs [89, 96, 98, 100]. The common dehydrogenase/reductase active site motif YXXXK was experimentally confirmed to be indispensable for FAR enzymatic activity [167]. Few studies have addressed the sequence determinants of FAR specificity. A study on FAR5 and FAR8 from *Arabidopsis*, which prefer C18 and C16 acyls, respectively, showed that reciprocal domain swaps and single amino acid mutations (355Ala/Leu and 377Val/Met) in the C-terminal part of the sequence resulted in a transition between C16 and C18 substrate preference [167].

Mutagenesis experiments performed with insect FARs also indicate that a limited number of amino acid substitutions can profoundly change the enzyme specificity [119, 120].

Currently, there is no publicly available protein structure of a FAR, which presents a challenge for performing further mutagenesis studies to infer the mechanisms of FAR specificity determination and to engineer FARs with novel or desired enzymatic properties.

4 Applications of FA-modifying enzymes

The potential biotechnological applications arising from the diverse enzymatic capabilities of insect FA-modifying enzymes are centered mainly on the synthesis of insect pheromones for pest management. However, there are also other, mostly unexplored potential applications for these insect enzymes (Figure 2).

Synthetic insect pheromones are used in a variety of pest management strategies in agricultural fields, forests, and urban areas to replace or complement traditional insecticides. The pheromones have several inherent advantages over insecticides: they are active in extremely small amounts (nanogram and sub-nanogram quantities), and they are specific toward the target species and generally non-toxic to other animals or humans (reviewed by Witzgall et al. [168]). Currently, synthetically prepared insect pheromones are used either as attractants for monitoring or mass trapping of insect pests, monitoring of other relevant insect species such as endangered species ([169], reviewed in [170]), or mating disruption of pest species via the release of synthetic sex pheromones that compromise olfactory communication and mate finding in insect pests [168].

The estimated total area of land treated with synthetic insect pheromones is approximately 10,000,000 ha worldwide and, for example, the global production of codlemone, the codling moth (*Cydia pomonella*) sex pheromone, reached 25,000 tons in 2010 [168]; it is primarily used in apple orchards. The pheromones have also been used widely in vineyards for mating disruption of the grapevine moth *Lobesia botrana* in Germany, Italy and California [168].

The establishment of economically viable synthesis of pheromones remains a major obstacle to scaling up the use of pheromone chemicals in pest control. In particular, FA-derived pheromone biosynthesis faces several challenging issues, such as the requirement for precisely positioning one or multiple double bonds into the synthesized FA-chain in a particular double bond configuration [171]. In this respect, employment of insect FA-modifying pheromone-biosynthetic enzymes or organisms heterologously expressing these enzymes may be a more economic and environmentally friendly option than traditional chemical synthesis.

The concept of producing pheromone chemicals in genetically modified plants ("pheromone farming") or yeasts ("pheromone brewing") has been tested [23, 25]. In these projects, the researchers semi-synthetically prepared moth pheromones by chemical reduction and consecutive acetylation of *Z*11-unsaturated FAs produced in genetically modified plants expressing moth *Z*11-mFAD. Ding et al. reconstructed a complete pheromone biosynthetic pathway by transforming the tobacco plant with a combination of two insect pheromone biosynthetic genes (mFAD and FAR) and two non-insect genes (thioesterase, which modified the length of de novo biosynthesized FAs, and acetyltransferase, which catalyzed the final

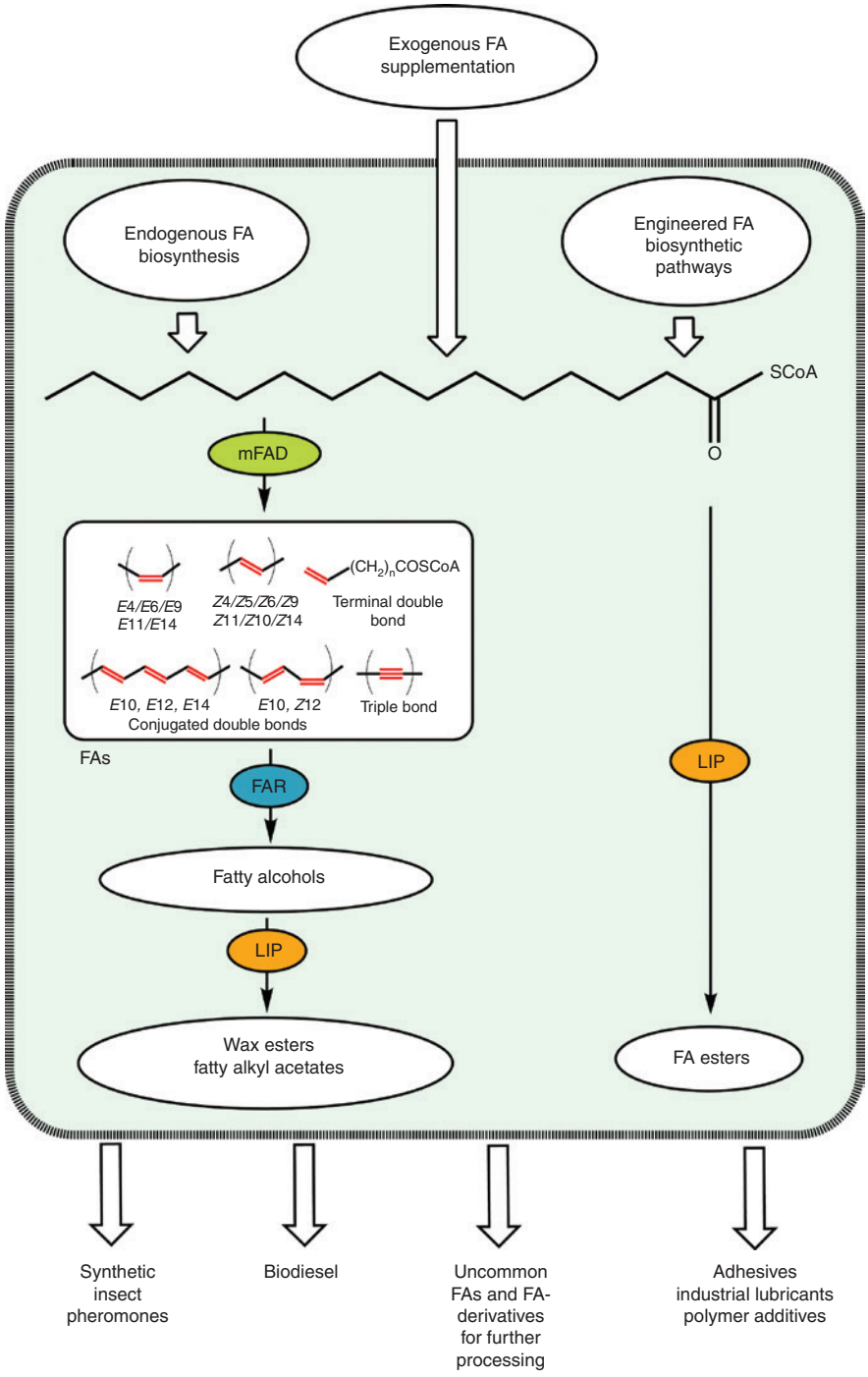

Figure 2: FA-modifying reactions that can be engineered in plant and microbial cells with the help of insect pheromone-biosynthetic enzymes. mFAD, fatty acyl desaturase; LIP, lipase; FAR, fatty acyl reductase; and FA, fatty acid.

pheromone biosynthetic step yielding the fatty alkyl acetates) [24]. The approach of combining insect and non-insect genes in the host organism is particularly useful in situations when suitable genes have not yet been isolated from insects. Plant "pheromone factories" possess another attractive feature: if proven non-harmful to the environment, they could be planted directly in fields, thus circumventing the necessity of isolating and dispersing pheromones from specialized dispensers for mating disruption [24].

The yeast *S. cerevisiae* is a frequently used host for metabolic pathway engineering [172] and represents a promising host organism for heterologous expression of FARs capable of producing biologically active

pheromone chemicals [118]. The yeasts co-expressing FAR and *Z*11-mFAD have been used to produce alcohol pheromone precursors that can be subsequently isolated and chemically oxidized to the biologically active aldehyde pheromones [23].

Muñoz et al. [173] tested another approach to small-scale pheromone synthesis, using immobilized bacterial acetyl transferase to catalyze the terminal step of the biosynthesis of a di-unsaturated conjugated fatty alkyl acetate, a cotton leafworm (*Spodoptera littoralis*) sex pheromone.

In a semi-synthetic approach, insect enzymes would be used to produce the precursors of compounds that are difficult to access solely by traditional organic synthesis tools, and these would be further chemically modified [23–25]. Such an approach could yield FAs with multiple double bonds in uncommon positions and geometric configurations or possibly molecules bearing more than one functional group. The insect FARs, with their strict substrate specificity toward uncommon substrates, could serve to efficiently and specifically convert non-conventional FAs into their respective alcohols.

In many cases, handling isolated enzymes is difficult due to their transmembrane character (e.g. mFADs) or requirement for additional protein partners and coenzymes. The only known exception among the FA-modifying enzymes is the lipases, which do not require a coenzyme, are usually soluble, and have been shown to be stable and active in a variety of different environments (including organic and ionic solvents) [174, 175]. The use of lipases from microorganisms and animals has been widely established on an industrial scale [176]; for example, more than 10,000 tons of pure 1-phenylethylamine enantiomer are produced annually using lipase as a catalyst [177]. Lipases are abundant in insect genomes. The majority of them evolved after divergence of insect orders [178], and thus, they potentially possess properties not present among lipases from other organisms. Insect lipase specificities and enzymatic properties, however, remain poorly studied in general. The isolated lipases from non-insects have been used for enantioselective synthesis of pheromones from bark beetles [179], corn rootworms [180], rice moths [181], and many other insect species [182].

To the best of our knowledge, there is currently no commercial application of insect enzymes in the synthesis of oleochemicals, although the implementation of some insect enzymes could substantially expand the toolbox of FA modifications available to both organic chemistry and mass industrial production. One recent example from the research field is the use of honeybee FAR1 for the synthesis of wax esters in engineered *S. cerevisiae* co-expressing FA elongase and wax synthase [183].

5 Challenges and future perspective

One of the main drawbacks to the use of genetically modified organisms for production of pheromones and other FA-derivatives is the cost of developing such transgenic organisms. Additionally, substantial optimization and scale-up of production capacity are required to convert the heterologous expression systems used in research into feasible production organisms. So far, efforts to produce insect pheromones in host organisms have led to (i) 2 mg/L of fatty alcohols in *S. cerevisiae* liquid culture [23]; (ii) 44 mg of unsaturated FA-derived methyl ester per kg of plant material [25]; and (iii) hundreds of mg of unsaturated FAs, tens of mg of the respective fatty alcohols, and several mg of the final acetates per kg of fresh leaf tissue from a *Nicotiana benthamiana* expression system [24]. In host systems transformed with non-insect enzyme sequences, the production of fatty alcohols and wax esters in bacteria (*E. coli* or Cyanobacteria) or yeasts (*S. cerevisiae*, *Rhodosporidium toruloides*, or *Yarrowia lipolytica*) can reach hundreds to thousands of mg/L cultivation medium after lab-scale optimization [7, 184–187]. In particular, oleaginous yeasts such as *R. toruloides* [188] and *Y. lipolytica* [6, 189–191], which accumulate large amounts of lipids and have established genetic engineering procedures, might be promising host organism candidates. As a future prospect, the production of desired pheromone chemicals might also be achieved through expression of enzymes with engineered functions.

There are multiple avenues potentially leading to enhanced yields of FA biosynthetic enzymes: (i) genetic modification of the ER retention signal [24], (ii) optimization of the expressed gene codon usage for the host organism [192], (iii) promoter strength and introduction of regulatory sequences such as the eukaryotic Kozak consensus sequence [193], (iv) moving from transient plant transformation and yeast expression plasmids toward stable plant transformants [24] and genome-integrated sequences in yeast [172], and (v) extensive metabolic engineering of the host organisms [7]. In addition to increasing the yield of specific FA derivatives, attention must also be paid to biosynthesis of side products such as undesired FA isomers, which could act as a repellent or inhibitor and thus compromise the pest management strategy.

This review has focused on a relatively minor part of insect biosynthetic capabilities. In a broader context, we envision that insect bioprospecting, the search among insect organisms for commercially valuable resources [194], eventually will exploit their genetic and biosynthetic diversity.

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