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Review

Reporter gene assays for screening and identification of novel molting hormone- and juvenile hormone-like chemicals

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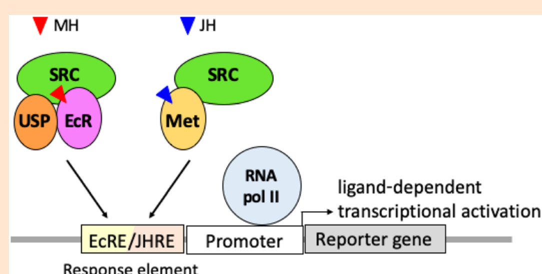

A reporter gene assay (RGA) is used to investigate the activity of synthetic chemicals mimicking the molting hormones (MHs) and juvenile hormones (JHs) of insects, so-called insect growth regulators (IGRs). The MH receptor, a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP), and the JH receptor Methoprene-tolerant (Met) are ligand-dependent transcription factors. Ligand-bound EcR-USP and Met bind to specific *cis*-acting DNA elements, referred to as the ecdysone-responsive element (EcRE) and the JH-responsive element (JHRE), respectively, in order to transactivate target genes. Insect hormone-induced transactivation systems have been reconstituted by the introduction of reporter genes under the control of EcRE and JHRE, or two-hybrid reporter genes, into insect, mammalian, and yeast cells expressing receptor proteins. RGA is easy to use and convenient for examining the MH- and JH-like activities of synthetic chemicals and is suitable for the high-throughput screening of novel structural classes of chemicals targeting EcR-USP and Met.

Keywords: reporter gene assay, ecdysone receptor, juvenile hormone receptor, agonist, insect growth regulator.

Introduction

Insect species that belong to a large class of Arthropoda utilize unique endocrine systems consisting of two peripheral hormones, molting hormones (MHs) and juvenile hormones (JHs). These hormones are important for regulating the growth and development of insects. The steroid 20-hydroxyecdysone (20E) and sesquiterpenoid JH III are a representative MH and JH, respectively. Synthetic nonsteroidal ecdysone agonists and juvenile hormone agonists (JHAs) have recently been developed as insecticides and categorized as insect growth regulators (IGRs).[1,2]

Since these synthetic insect hormones interfere with insect-specific hormone responses, they are considered to be safer for mammals than other classes of insecticides.[1] Nonsteroidal ecdysone agonists exhibit insect-selective toxicity for certain taxonomic orders. These IGRs may be ideal compounds for pest control.[1,3,4]

The activities of synthetic MHs and JHs were previously analyzed in the whole body using classical *in vivo* bioassay methods.[5–11] Experimental procedures to examine hormone-dependent morphological changes and the stimulation/inhibition of proliferation using cultured insect cell lines have also been developed as *in vitro* bioassays.[12–18] The measurement of ligand-receptor binding affinity using insect cell extracts or *in vitro* translated receptor proteins is another procedure for detecting natural and synthetic hormones.[19–28] In the 1990s, genes encoding the ecdysone receptor (EcR) and its partner protein ultraspiracle (USP), a homolog of mammalian retinoid X receptor (RXR), and the JH receptor Methoprene-tolerant (Met) were initially identified in the fruit fly *Drosophila melanogaster*.[29–32] EcR-USP and Met are ligand-dependent transcription factors belonging to the nuclear receptor (NR) superfamily and the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) family, respectively (Fig. 1a and Fig. 4a). The genes encoding EcR-USP and Met are conserved among various insect species and have

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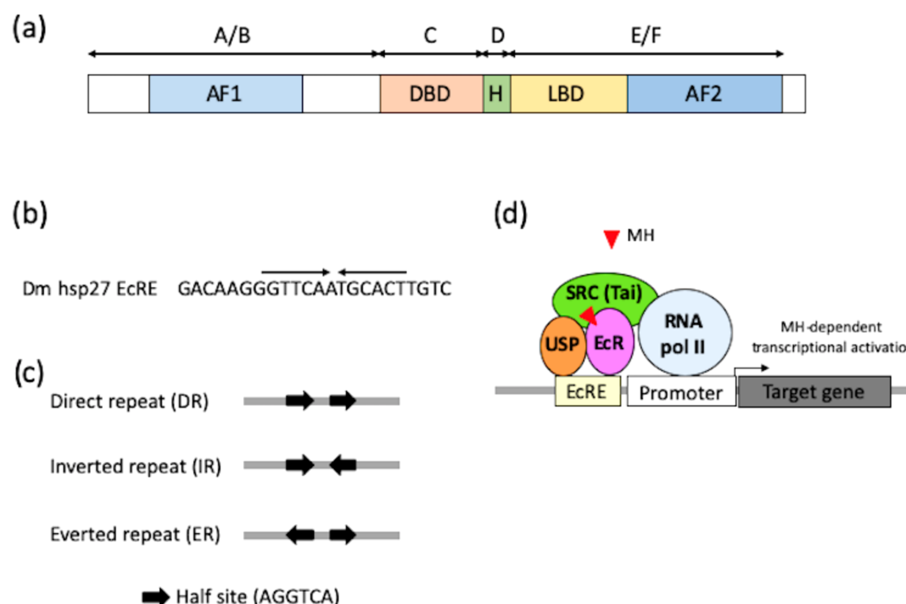

Fig. 1. MH-receptor EcR. (a) Domain structure of EcR. NR superfamily proteins including EcR are comprised of six domains (A–F) with conserved functions and sequences. AF: activation function; DBD: DNA-binding domain; H: hinge domain; LBD: ligand-binding domain.[136] (b) Nucleotide sequence of *D. melanogaster hsp27* EcRE.[43] The arrows indicate half-site sequences forming a putative palindromic structure. (c) Patterns of typical REs for vertebrate NRs. Half sites with a canonical sequence (AGGTCA) are aligned in three different orientations: inverted repeat (IR), direct repeat (DR), and everted repeat (ER). Two half sites are zero to several nucleotides apart.[137,138] (d) Schematic representation of the molecular mechanism underlying the MH-dependent transactivation of target genes by EcR-USP in *D. melanogaster*. See the text for details.

been functionally characterized.[33–35)]

The reporter gene assay (RGA) is a versatile experimental method for monitoring gene expression associated with signal transduction cascades in response to intra- and extracellular stimuli. RGA has been employed to elucidate the molecular mechanisms of signal transduction *via* EcR-USP and Met, and it has also played important roles in the development of high-throughput screening systems for IGRs with MH- and JH-like activities. In this review, we summarized the application of RGA to the study of IGRs.

1. RGA

The term *reporter gene* refers to a gene encoding a protein product with unique and readily measurable enzymatic activity or that is distinguishable from a large amount of various proteins expressed in cells.[36–38] The following reporter genes are frequently and commonly used: genes for bacterial chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), and β-glucuronidase (GUS); luciferase from bacteria, firefly, or *Renilla*; green fluorescent protein (GFP) of jellyfish; and the secretable form of alkaline phosphatase (SEAP) derived from the human placenta.[36–40] These reporter gene products have been characterized in detail, and their advantages and disadvantages as well as detection methods have been reviewed elsewhere.[36,39,40]

Reporter genes are placed downstream of promoters that function in host cells. RGA is a versatile experimental method for examining various cellular events. The reporter gene may be used as a marker of gene transfer or in promoter analysis, the identification of *cis*- and *trans*-acting DNA elements of genes of interest, the spatiotemporal imaging of gene expression patterns, the characterization of receptors and their ligands, and analyses of signaling pathways.[41]

2. Molecular mechanisms underlying the activation of EcR-USP by MHs

The RGA procedure has been applied in many studies to investigate the molecular mechanisms underlying signal transduction initiated by MHs. Riddihough *et al.* reported that the transcription of the *D. melanogaster* heat shock protein 27 (*hsp27*) gene was induced by ecdysone as well as heat shock. A deletion analysis of the *hsp27* promoter identified ecdysone-specific *cis*-acting DNA elements, referred to as the ecdysone-responsive element (EcRE), with a putative palindrome structure (Fig. 1b).[42,43] A gene for the MH receptor EcR was cloned as the first member of the NR superfamily in *D. melanogaster*. The EcR protein was shown to bind to active MH 20E as well as DNA with high specificity at EcRE.[29] MH-bound EcR forms a heterodimer with its partner protein, USP, on EcRE.[30,31,44] Furthermore, the steroid receptor coactivator (SRC), Taiman of *D. melanogaster* (DmTai), was identified as an important component in the transduction of MH signals to downstream effectors.[45]

EcREs with distinct sequences were subsequently identified in the regulatory region of *D. melanogaster Fbp1*, *Eip28/29*, and *Aedes aegypti* vitellogenin (Vg) genes.[46–49] Previous studies also indicated that EcR-USP induced reporter gene expression *via* asymmetric RE, a direct repeat of the core sequence (half site) in African green monkey CV-1 cells.[50,51] The heterodimeriza-

Table 1. *In vitro* RGA systems for the screening of EcR-USP ligands

Host cells	Organism and cell lines	Transfected receptor genes	EcRE	Reporter gene	Types of test compounds	Ref.
Insect cells*	*Drosophila melanogaster* Kc	—	Dm hsp27 promoter	Luc	20E, α-ecdysone	55)
	D. melanogaster Kc	—	Dm hsp27 promoter	Luc	3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide (DTBHIB)	56)
	D. melanogaster Kc	—	Dm hsp27 promoter	Luc	Diacylhydrazine (DAH) compounds	57)
	D. melanogaster Schneider 2 (S2)	—	Dm hsp27 EcRE	*lacZ*	Cucurbitacins	134)
	D. melanogaster Schneider SL2	—	Dm hsp27 EcRE	Luc	Insect and plant ecdysteroids	139)
	Spodoptera frugiperda Sf9	—	Dm hsp27 EcRE	Luc	20E, ponasterone A (pon A), chromafenozide	67)
	D. melanogaster Kc	—	Dm hsp27 EcRE	Luc	20E, pon A, chromafenozide	67)
	Bombyx mori Bm5	—	Dm hsp27 EcRE	CAT GFP	Plant compounds DAH library	58)
	S. frugiperda Sf9	CfEcR	Dm hsp27 EcRE	CAT	RG-102240	68)
	Lymantria dispar (Ld652Y)	CfEcR MmRXR	GAL4 RE (two-hybrid)	CAT GFP	RG-102240	68)
	B. mori Bm5	—	Dm hsp27 EcRE	GFP	DAHs	70)
	D. melanogaster S2	—	Dm hsp27 EcRE	Luc	Chromafenozide Methoxyfenozide	69)
	B. mori Bm5	—	Dm hsp27 EcRE	Luc	Chromafenozide Methoxyfenozide	69)
	Anthonomus grandis BRL-AG-3A BRL-AG-3C	—	Dm hsp27 EcRE	Luc GFP	DAHs	76)
	Leptinotarsa decemlineata BCRL-Lepd-SL1	—	Dm hsp27 EcRE	Luc GFP	DAHs	76)
	D. melanogaster S2	—	Dm hsp27 EcRE	CAT Luc	DAHs, acylaminoketone (AAK) analogs, tetrahydroquinoline (THQ) compounds, piperidiamine analogs	80)
	B. mori Bm5	—	Dm hsp27 EcRE	GFP	DAHs, AAK analogs, THQs	71)
	S. littoralis Sl2, Slj2b	—	Dm hsp27 EcRE	Luc	DAHs, AAK analogs, THQs	71)
	S. frugiperda Sf9	—	Dm hsp27 EcRE	Luc	Non-steroidal compounds	78)
	L. decemlineata BCRL-Lepd-SL1	—	Dm hsp27 EcRE	Luc	DAHs	77)
	D. melanogaster S2	—	Dm hsp27 EcRE	Luc	Ecdysteroids	135)
	B. mori Bm5	—	Dm hsp27 EcRE	Luc	Ecdysteroids	135)
	S. frugiperda Sf9	—	Dm hsp27 EcRE	Luc	*N*-*tert*-butylphenyl thenoylhydrazide compounds	140)
	D. melanogaster S2	—	Dm hsp27 EcRE	Luc	Compounds registered in Maybridge database	79)
	B. mori Bm5	—	Dm hsp27 EcRE	Luc	Compounds registered in Maybridge database	79)
Mammalian cells	*Cricetulus griseus* CHO	VgEcR HsRXR	E/GRE	*lacZ*	Phytochemicals	90)
	Mus musculus NIH 3T3	AaEcR MmRXR	GAL4 RE (two-hybrid)	Luc	THQs	3)

Table 1. Continued

Host cells	Organism and cell lines	Transfected receptor genes	EcRE	Reporter gene	Types of test compounds	Ref.
Mammalian cells	*Homo sapiens* HEK293	BmEcR	Dm hsp27 EcRE	β-gal	AAKs	72)
	H. sapiens HEK293	BmEcR	Dm hsp27 EcRE	β-gal	AAKs	60)
	C. griseus CHO	CfEcR HsRXR-LmRXR chimera	GAL4 RE (two-hybrid)	Luc	AAKs	60)
	M. musculus NIH 3T3	CfEcR LmRXR DmTai	GAL4 RE (two-hybrid)	Luc	DAHs, THQs	61)
	M. musculus NIH 3T3	AaEcR CfEcR DmEcR MsEcR BmEcR LmEcR TmEcR AmaEcR HsRXR	GAL4 RE (two-hybrid)	Luc	THQs	4)
	M. musculus NIH 3T3	AaEcR CfEcR DmEcR MsEcR BmEcR TmEcR AmaEcR BaEcR NcEcR HsRXR	GAL4 RE (two-hybrid)	Luc	Natural and semi-synthetic ecdysteroids (hydroxylation)	62)
	M. musculus NIH 3T3	VgEcR HsRXR	E/GRE	Luc	Natural and semi-synthetic ecdysteroids (hydroxylation)	62)
	M. musculus NIH 3T3	AaEcR CfEcR DmEcR HsRXR-LmRXR chimera	GAL4 RE (two-hybrid)	Luc	Natural and semi-synthetic ecdysteroids (alkylation)	63)
	M. musculus NIH 3T3	VgEcR HsRXR	E/GRE	Luc	Natural and semi-synthetic ecdysteroids (alkylation)	63)
	H. sapiens HEK293	BmaEcR HsRXR	GAL4 RE (two-hybrid)	Luc	Steroidal ecdysones, DAHs	64)
Yeast	*Saccharomyces cerevisiae*	CfEcR-CfUSP HsRXR MmGRIP1	Dm hsp27 EcRE	β-gal	Nonsteroidal ecdysone agonists	85)
	S. cerevisiae	AaEcR-AaUSP DmUSP CfUSP HsRXR MmGRIP1	Dm hsp27 EcRE	β-gal	Ecdysteroids, nonsteroidal ecdysone agonists	84)
	S. cerevisiae	DmEcR-DmUSP CsEcR-CsUSP LdEcR-LdUSP DmTai	IR0 DR2 DR4	β-gal	Ecdysteroids, DAHs, THQ	87)

Abbreviations Aa: *Aedes aegypti*; Ama: *Amblyomma americanum*; Ba: *Bemisia argentifolii*; Bm: *Bombyx mori*; Bma: *Brugia malayi*; Cf: *Choristoneura fumiferana*; Cs: *Chilo suppressalis*; Dm: *Drosophila melanogaster*; E/GRE: ecdysone/glucocorticoid responsive element (hybrid RE); Hs: *Homo sapiens*; Lm: *Locusta migratoria*; Ms: *Manduca sexta*; Nc: *Nephotettix cincticeps*; Tc: *Tribolium castaneum*; Tm: *Tenebrio molitor*; Mm: *Mus musculus*; CHO; Chinese hamster ovary; GAL4 RE: DNA element for binding of the GAL4 DNA-binding domain (DBD); VgEcR: hybrid of *D. melanogaster* EcR carrying modified DBD and VP16 AD that recognizes an E/GRE *Reporter gene assays in insect cells expressing endogenous EcR and USP. Only the reporter plasmid was introduced.

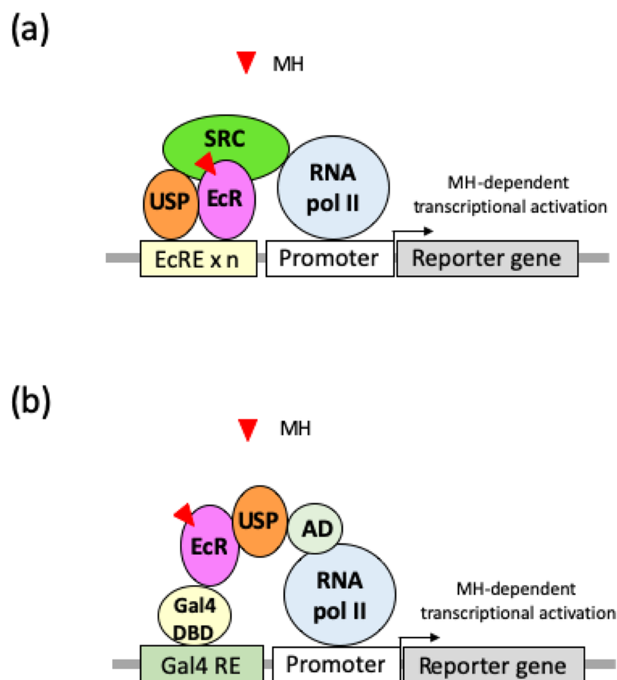

Fig. 2. RGA for EcR-USP. (a) Schematic summary of RGA for EcR-USP. RGA using a reporter gene under the control of EcRE, in which multiple copies of EcRE are integrated upstream of the promoter (EcRE×n). Ligand-bound EcR and USP form a heterodimer on EcRE. The interaction of transcriptional coactivator SRC enhances reporter gene expression by recruiting RNA pol II general transcription factors of host cells. (b) Two-hybrid-based RGA for EcR-USP. EcR and USP (LBD or full length) fused to Gal4-DBD and transcriptional activation domain (AD) of Gal4 or VP16, respectively (and *vice versa*), interact in an MH-dependent manner and induce reporter gene expression in host cells.

tion of EcR and USP on DR-RE (Fig. 1c) was confirmed using a biochemical approach, the electrophoretic mobility shift assay (EMSA).[52] The activation mechanism of EcR-USP revealed in *D. melanogaster* was conserved in other insect species (Fig. 1d).[33,50,53,54]

3. RGA for detecting Synthetic ecdysone agonists by surveying gene expression mediated by EcR-USP

The identification of EcRE drove researchers to develop RGA systems with the capacity to detect synthetic ecdysone agonists by examining EcR-USP-mediated gene expression that enables activity-based high-throughput screening. RGA systems previously developed for EcR-USP in various host cells are summarized in Table 1. The basis for using RGA to monitor the ligand-dependent activation of EcR-USP-mediated gene expression is the construction of a reporter plasmid that results in the expression of reporter genes, including CAT, β-gal, Luc, and GFP, under the control of EcRE (Fig. 2a). Dm *hsp27* EcRE is recognized as an effective EcRE, and it is used in the development of a large number of RGA systems, regardless of host cell species (Table 1). The Dm *hsp27* promoter (~650 bps), or the fusion of a short oligonucleotide containing Dm *hsp27* EcRE with the basal promoters of insect genes, such as *D. melanogaster* alcohol dehydrogenase (ADH) and *Bombyx mori* actin A3 (ACT3), has frequently been used to express reporter genes in insect cell lines.[29,55–58] In mammalian RGA, Dm *hsp27* EcRE was connected to minimal promoters of thymidine kinase (TK) or mouse mammary tumor virus (MMTV).[30,31,59] Ligand-bound EcR and USP expressed from the insect genome (endogenous EcR-USP) or transfected genes (in mammalian or yeast cells) bind to the EcRE of the reporter plasmid and then induce the transcription of reporter genes instead of native target genes (Fig. 2a). Two-hybrid-based RGAs are often used to examine ligand-dependent interactions between EcRs and USPs in heterologous hosts (Fig. 2b and Table 1). The heterodimerization of ligand-bound EcR and USP enables the transactivation of two-hybrid reporter genes.[3,4,60–64]

Pioneering studies using RGA on insect cell lines were conducted by Mikitani, who introduced a Luc reporter plasmid connected to a Dm *hsp27* promoter into *D. melanogaster* Kc cells that were highly sensitive to ecdysteroids. The ecdysteroid-dependent expression of luciferase by endogenous EcR-USP was reproducibly observed. This procedure was very sensitive, rapid, and simple, suggesting that RGA of insect cells is a valuable tool in the search for new ecdysteroid compounds.[55] The findings obtained using RGA correlated with the effects of synthetic ecdysteroid compounds on ligand-binding affinity, efficacy toward cellular morphological changes, and larvicidal activity.[56–58] To date, a number of RGA systems for EcR-USP have been developed in several cell lines from insect species in distinct phylogenetic orders.

RH5849, a diacylhydrazine (DAH)-type lead compound exhibiting MH-like activity, was identified as the first nonsteroidal ecdysone agonist.[65,66] DAHs exhibited *in vivo* toxicity in a lepidopteran insect–selective manner, which correlated with the transactivation activity observed in RGA.[58,65–71] α-Acylaminoketone and its analogs were identified as novel types of nonsteroidal ecdysone agonists with potent selectivity on lepidopteran EcR-USP, while tetrahydroquinoline (THQ) compounds were shown to strongly activate dipteran EcR-USP.[3,4,60,61,71,72] The insect order-selective activity of nonsteroidal ecdysone agonists was associated with the divergence of the primary sequence of the ligand-binding domain (LBD) of EcR. Differences in the three-dimensional (3D) structure of the ligand-binding pocket affected the binding potency of nonsteroidal ecdysone agonists; however, the binding mode of natural ecdysteroids was similar among different phylogenetic orders of insects.[73–75]

To discover new classes of ecdysone agonists for non-lepidopteran insect pests, RGA was established in coleopteran cell lines from *Leptinotarsa decemlineata* and *Anthonomus grandis*, and various nonsteroidal ecdysone agonists were tested by methods combined with (quantitative) structure–activity relationship ((Q)SAR) studies and/or the 3D-modeling of the ligand-binding pocket and ligand docking studies (virtual screening).[71,76,77] Similarly, the agonist/antagonist activities for dipter-

an and lepidopteran EcR-USP of several compounds identified by ligand-based virtual screening were confirmed by RGA.[78,79] Approaches combining (Q)SAR and RGA were also used to examine the ligand potencies of semi-synthetic ecdysteroids for EcR-USP of various insect species.[62,63]

In mammalian cell-based RGA, EcR-USP derived from several different orders of insects was expressed to examine the MH-like activity of test compounds.[4,62,63] RGA was also utilized to compare the species-selective *in vivo* toxicities and *in vitro* transactivation activities of synthetic compounds in the lepidopteran insects *B. mori* and *Spodoptera littoralis*.[80]

4. Reconstitution of MH-induced transactivation systems in yeast

RGA has been established in the yeast *Saccharomyces cerevisiae* as another heterologous host system. *S. cerevisiae* is the simplest eukaryote possessing highly conserved gene expression mechanisms of higher eukaryotes.[81] Previous studies reported that the ligand-dependent transactivation activity of *D. melanogaster* EcR-USP *via hsp27* EcRE was not confirmed, whereas the EcR protein expressed in yeast was capable of binding to ecdysteroid ligands with the co-expression of USP.[82,83] Tran *et al.* showed that the expression of mouse SRC GRIP1 was required to reconstitute the ligand-dependent transactivation of EcR-USP from *Choristoneura fumiferana* and *Aedes aegypti*.[84,85] The ligand-induced heterodimerization of the LBDs of EcR and USP from *D. melanogaster* was also demonstrated by a two-hybrid method and EMSA in yeast.[86] Based on these findings, we recently established a new yeast RGA system for detecting MHs.[87] EcR and USP derived from three insect species belonging to different taxonomic orders—the dipteran *D. melanogaster*, lepidopteran *Chilo suppressalis*, and coleopteran *L. decemlineata*—were expressed in yeast in conjunction with the SRC DmTai (Fig. 3). By optimizing response elements, yeast RGA exhibited marked responses and detected EcR ligands in dose-dependent and ecdysteroid-specific manners. The insect order-selective ligand activities of the nonsteroidal ecdysone agonists, DAHs and THQ, for lepidopteran and dipteran EcRs, respectively, were also detectable. The potencies of these ligands in yeast RGA linearly correlated with their binding activities.[87]

Heterologous RGAs established in mammalian or yeast cells tended to exhibit lower ligand responses than those in insect cells.[85,87,88] In mammalian RGAs, chimeric EcR (VgEcR), in which *D. melanogaster* EcR carrying modified DBD was fused to VP16 AD, was constructed in order to achieve robust responses.[89] VgEcR recognizes hybrid response elements containing ecdysone and glucocorticoid responsive element (E/GRE)[89] and has been used in some mammalian RGAs.[62,63,90] In yeast RGA, the genetic manipulation of the host yeast strain was effective for improvement; the deletion of plasma membrane-localized efflux pumps and/or cell wall mannoproteins resulted in increased ligand responses.[85,91,92] Heterologous host cells that lack ecdysteroid-regulated gene expression systems, however, are considered to be suitable for examining EcR-USP-ligand interactions without perturbation by endogenously expressed EcR-USP.[93] Unlike mammalian cells that express RXRs, functional homologs of insect USPs,[30,31,84,85] yeast does not possess NR homologs.[94] Yeast RGA, which is a rapid, easy, and cost-effective procedure, may be advantageous for directly measuring the MH-like activities of synthetic compounds on EcR-USP.

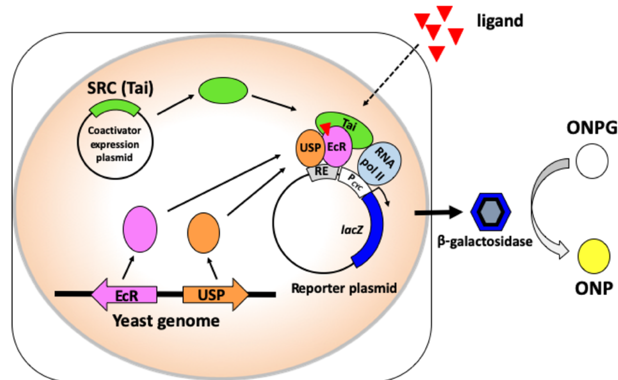

Fig. 3. Principle of RGA in yeast strains expressing insect ecdysone receptors EcR and USP. In response to ligands, EcR and USP expressed in yeast cells form a heterodimer and bind to response elements upstream of the minimal promoter of the *iso*-cytochrome c (CYC) gene. *D. melanogaster* Tai, a steroid receptor coactivator (SRC), cooperates with EcR-USP to enhance the expression of the *lacZ* reporter gene by recruiting the RNA pol II general transcription factors of yeast. The expression of *β*-galactosidase may be visualized and quantified by the development of a yellow color due to the accumulation of ONP in the assay buffer. ONPG: *o*-nitrophenyl-*β*-D-galactopyranoside; ONP: *o*-nitrophenol; RNA pol II: RNA polymerase II; P_{CYC}: minimal promoter of the CYC gene.[87]

5. Molecular mechanisms underlying JH-dependent transactivation in arthropods

JH receptor Met proteins are members of the bHLH-PAS family that function as ligand-dependent transcription factors as well as EcR-USP (Fig. 4a).[34,35] The Met gene was originally cloned as a gene that complemented the methoprene resistance of a mutant fly.[32] The Met protein was shown to bind to JH III with high affinity. The GAL4-DBD-Met fusion protein directed to GAL4 RE transactivated the Luc reporter in a JH/JHA-dependent manner.[24] Met homologs have since been identified in various insects, such as holo-, hemi-, and ametabolous species.[26,95–97] The paralogous gene *germ cell–expressed* (*Gce*), which is conserved in *Drosophila* species, is also involved in signal transduction for the exertion of JH effects.[98–100] Met-Met and Met-Gce dimer complexes are formed as inactive states in the absence of JHs. Upon ligand binding to the PAS-B domain of Met and Gce,[26] Met-Met and Met-Gce complexes are rapidly dissociated.[101] Met/Gce proteins then form a heterodimer with other bHLH-PAS protein SRCs, such as DmTai, *A. aegypti* FISC, and *Tribolium castaneum* TcSRC.[102,103] The Met-SRC and Gce-SRC heterodimer complex binds to a specific DNA element (JHRE) in a JH-dependent manner and activates the transcription of early JH-inducible genes, such as Krüppel homolog 1 (*Kr-h1*) and early trypsin (*ET*) (Fig. 4b).[102–104] Previous studies

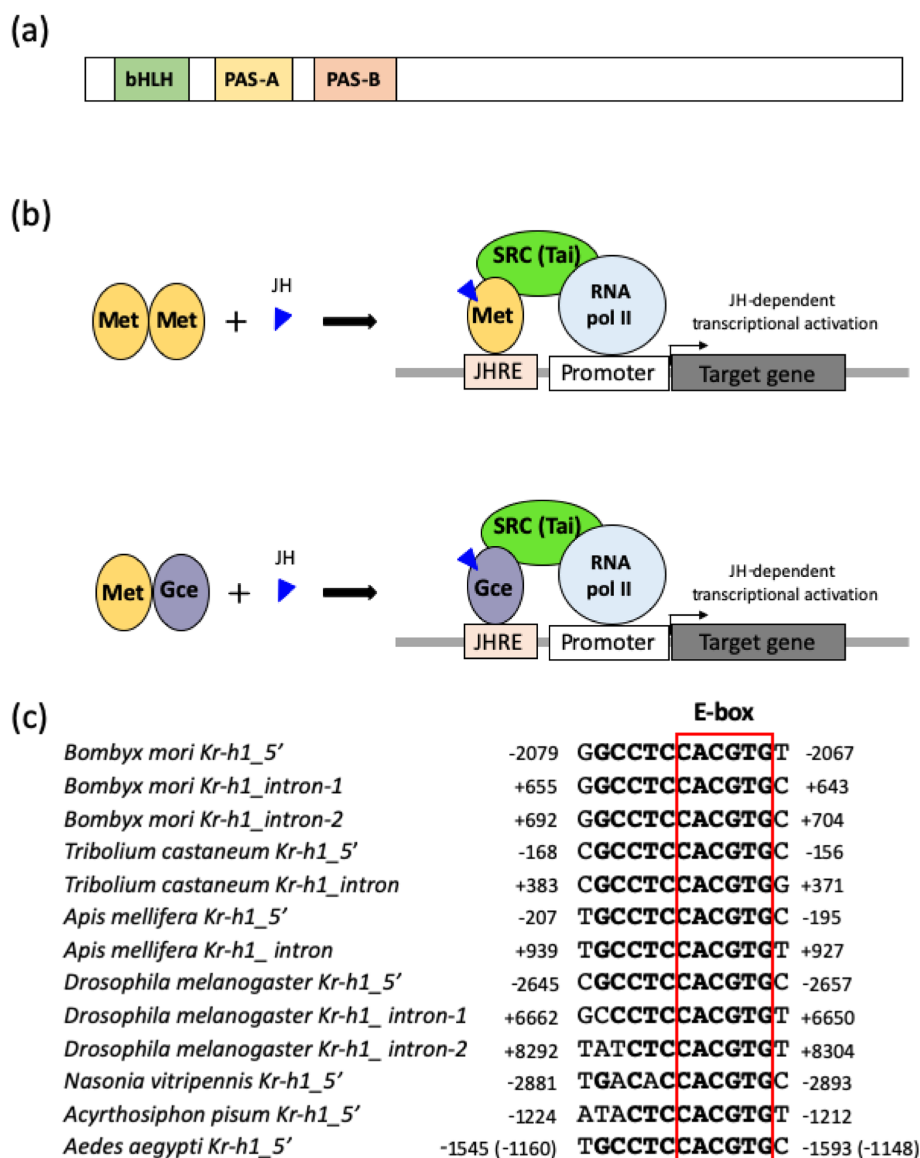

Fig. 4. JH-receptor Met. (a) Domain structures of Met and Gce, members of the bHLH-PAS domain protein family. Met and Gce are comprised of three functional domains—bHLH: a basic helix-loop-helix domain involved in DNA binding; PAS-A: a domain for transcriptional activation; PAS-B: a domain for ligand binding.[26,116] (b) Schematic representation of the JH-dependent transactivation of a target gene by Met/Gce in *D. melanogaster*. See the text for details. (c) Conservation of the JHRE motif including the E-box in the regulatory regions of the *Kr-h1* gene of various insects.[105–107]

on the expression of anti-metamorphic gene *Kr-h1* identified a 13-nucleotide motif containing an E-box (CAC GTG) as JHRE, which was essential for the binding of Met-SRC to mediate the effects of JH. This motif is highly conserved in the *Kr-h1* regulatory region of a wide range of insect species (Fig. 4c).[105–107] In *A. aegypti*, Met and Cycle (Cyc) have been shown to form a heterodimer on response elements containing an imperfect E-box-like (CAC GCG) sequence in female mosquitos. The Met-Cyc complex induces the JH-dependent expression of the *Kr-h1* gene, as well as the Met-FISC heterodimer.[108]

JH-regulated gene expression is shared in microcrustacea *Daphnia pulex* and *D. magna*, in which Met and SRC form a heterodimer in response to various juvenoids.[109] Juvenoids are involved in environmental sex determination and induce male offspring production in female populations of daphnids.[110–112] Although Met and SRC play essential roles in normal embryogenesis,[109] the expression of *D. pulex Kr-h1* is not regulated by the JH-Met signaling cascade, as it is in insects.[113]

6. RGA systems for detecting JHs and JHAs

Table 2 summarizes RGAs previously established in studies on JH receptors. The strategy of constructing reporter plasmids is similar to that of constructing RGAs for EcR-USP: DNA fragments containing JHRE identified in genes such as *Kr-h1*, juvenile hormone esterase (*jhe*), and early trypsin (*ET*) were integrated upstream of basal promoters (Table 2).[27,28,105–107,114–117]

Table 2. RGA systems for arthropod JHRs

Host cells	Organisms and cell lines	Transfected receptor genes	Response element	Reporter gene	Ref.
Insect cells	*C. fumiferana* CF-203*	—	C*fjhe* JHRE	Luc	114)
	D. melanogaster S2	DmMet	GAL4 RE (one-hybrid)	Luc	24)
	D. melanogaster L57	AaMet-AaFISC	GAL4 RE (two-hybrid)	Luc	102)
	D. melanogaster L57*	—	Aa*ET* JHRE	Luc	102)
	B. mori NIAS-Bm-aff3*	—	Bm*Kr-h1* JHRE	Luc	105)
	Tribolium castaneum Tc81*	—	Tc*Kr-h1* JHRE	Luc	106)
	D. melanogaster S2	TcMet-TcSRC	Tc*Kr-h1* JHRE	Luc	106)
	Aedes aedypti Aga2*	—	Synthetic AaMFBS	Luc	104)
	Aedes aegypti Aga2*	—	Aa*Kr-h1* JHRE	Luc	107)
	D. melanogaster Kc*	—	Dm*Kr-h1* JHRE	Luc	115)
	D. melanogaster S2	BmMet-BmSRC	Bm*Kr-h1* JHRE	Luc	116)
	D. melanogaster S2	DmMet/Gce-DmTai	Aa*ET* JHRE	Luc	27)
	D. melanogaster S2*	—	Aa*ET* JHRE	Luc	28)
Mammalian cells	*M. musculus* NIH 3T3	TcMet-TcSRC	GAL4 RE (two-hybrid)	Luc	103)
	H. sapiens HEK293	BmMet2-BmSRC	GAL4 RE (two-hybrid)	Luc	105)
	H. sapiens HEK293	BmMet2-BmSRC	Bm*Kr-h1* JHRE	Luc	105)
	H. sapiens HEK293	TcMet-TcSRC	GAL4 RE (two-hybrid)	Luc	106)
	C. griseus CHO	DapmaMet-DapmaSRC DappuMet-DappuSRC	GAL4 RE (two-hybrid)	Luc	109)
	H. sapiens HEK293	BmMet2-BmSRC BmMet1-BmSRC	Bm*Kr-h1* JHRE	Luc	116)
	H. sapiens HEK293	BmMet1-BmSRC	GAL4 RE (two-hybrid)	Luc	116)
	C. griseus CHO	DapmaMet-DapmaSRC	GAL4 RE (two-hybrid)	Luc	119)
	C. griseus CHO	DappuMet-DappuSRC TcMet-TcSRC AaMET-AaFISC DmMet-DmTai DmGCE-DmTai	GAL4 RE (two-hybrid)	Luc	120)
	C. griseus CHO	DappuMet-DappuSRC	Tc*Kr-h1* JHRE	Luc	118)
	H. sapiens HEK293T	DmMet-DmTai DmGCE-DmTai	GAL4 RE (two-hybrid)	Luc	28)
	H. sapiens HEK293T	DmMet-DmTai	Bm*Kr-h1* JHRE	Luc	117)
Yeast	*S. cerevisiae*	AaMet-AaCYC AaMet-AaFISC	GAL4 RE (two-hybrid)	*β*-gal	122)
	S. cerevisiae	AaMet-AaFISC	GAL4 RE (two-hybrid)	*β*-gal	123)

Abbreviations JHR: juvenile hormone receptor, Aa: *Aedes aegypti*; Bm: *Bombyx mori*; Cf: *Choristoneura fumiferana*; Dm: *Drosophila melanogaster*; Tc: *Tribolium castaneum*; Dapma: *Daphnia magna*; Dappu: *Daphnia pulex*; jhe: juvenile hormone esterase; ET: early trypsin; MFBS: Met-FISC binding site
* Reporter gene assays in insect cells expressing endogenous JHRs and a transcriptional coactivator. Only the reporter plasmid was introduced.

Ligand-bound Met heterodimerizes with SRC and binds to JHRE in order to transactivate reporter gene expression (Fig. 5a). The JH-dependent expression of reporter genes by Met and/or Gce has been measured in various insect cell lines (Table 2).[27,102,104–107,114–116] Although two-hybrid-based RGAs have been used frequently to examine JH-dependent heterodimer formation between Met and SRC in heterologous host systems (Fig. 5b), insect *Kr-h1* JHRE-regulated transactivation was successfully reconstituted using mammalian cells in three studies (Table 2).[105,117,118] Some of these RGAs indicated dose-dependent responses against various juvenoids, such as JH III, methyl farnesoate, methoprene, pyriproxyfen, and fenoxycarb.[27,105,109,117,119,120]

RGAs for Met from *Daphnia* species also have been established.[109,119,120] *D. pulex* Met exhibited dose-dependent responses against several juvenoids *via T. castaneum Kr-h1* JHRE,[118] while the *Kr-h1* homolog of *D. pulex* was not regulated by JH-Met signaling.[113] This finding suggests that the DNA-binding properties of Met proteins are conserved in insects and daphnids. RGA for Met derived from non-insect species will be use-

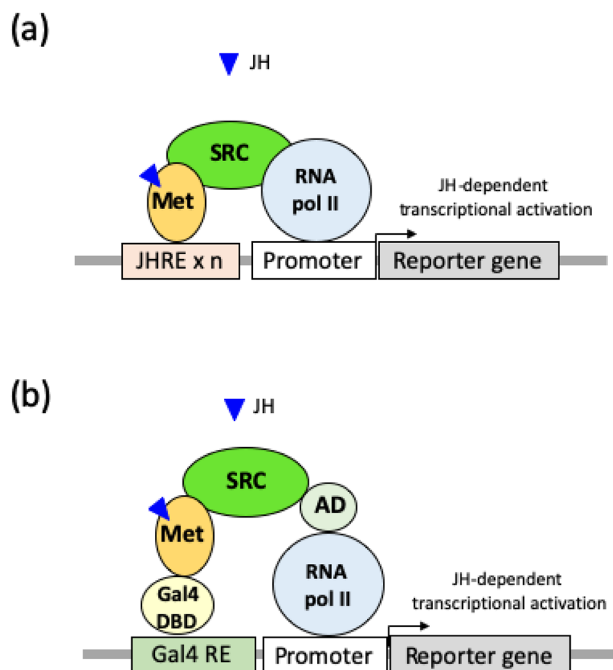

Fig. 5. RGA for Met. (a) Schematic summary of RGA for Met. RGA using a reporter gene under the control of JHRE, in which multiple copies of JHRE are integrated upstream of the promoter (JHRE×n). Ligand-bound Met and SRC form a heterodimer on JHRE, recruiting RNA pol II general transcription factors of host cells for the induction of the reporter gene. (b) Two-hybrid-based RGA for Met. Met and SRC (partial or full length) fused to Gal4-DBD and AD, respectively (and vice versa), interact in a JH-dependent manner and induce reporter gene expression in host cells.

ful for examining the influence of JHAs in environmental assessments.

RGA is valuable for revealing structural features in the binding between JHs/JHAs and Met. Bittova *et al.* examined the stereoselectivity and binding mechanisms of *D. melanogaster* Gce by RGA combined with a molecular modeling approach.[28] Yokoi *et al.* investigated the SAR of 14 natural and synthetic JHAs using their newly established RGA in HEK293T cells.[117] Unlike synthetic EcR ligands, JHAs with potent insect selectivities have not yet been developed.[121] However, a mutational analysis of the PAS-B domain identified two amino acid residues crucial for the ligand selectivity of Met proteins between insects and daphnids.[109,120] An analysis of structural aspects of the ligand-binding pocket of Met may facilitate the development of JHAs specific to insect pests.

Two-hybrid-based yeast RGAs expressing *A. aegypti* Met and FISC/CYC were also established. These RGAs were used to screen for plant-derived compounds with agonist or antagonist activities.[122,123]

Conclusion

Since synthetic ecdysone agonists and JHAs act on insect-specific endocrine systems, extensive efforts have been made to obtain novel compounds.[1,121] Regarding ecdysone agonists, derivatives of imidazothiadiazole,[124] 1-phenyl-4-cyano-5-aminopyrazoles,[125] heptacyclic pyrazolamide,[126] methylene lactams,[127] oxadiazolines,[128] and imidazoles[129] have been lined up by (Q)SAR studies and/or virtual screening methods. These compounds have potential as candidates of new classes of EcR-targeting insecticides. An *in silico* screening method was also used to obtain novel bioactive juvenoids.[130] Previous studies have reported several thousand synthetic compounds with JH-like effects, and chemically divergent compounds act as juvenoids.[131–133] Therefore, a novel class of JHAs, ideally insect-selective juvenoids, may be obtained in the future. Furthermore, RGA can confirm the antagonist activity of chemicals of interest.[58,78,79,90,122,123,134,135] In comparison with agonist screening, however, the general cytotoxicity of test chemicals that may repress reporter gene expression in a receptor-independent manner should be carefully considered in antagonist assays.[80] RGA using a specifically engineered reporter gene construct to positively select for a decrease in receptor activity may allow for the effective screening of antagonists from a large pool of chemical compounds. RGA systems are suitable and advantageous for the high-throughput screening of chemicals that affect the activity of EcR and Met.

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References

1) T. S. Dhadialla, G. R. Carlson and D. P. Le: New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* **43**, 545–569 (1998).
2) H. Tunaz and N. Uygun: Insect growth regulators for insect pest control. *Turk. J. Agric. For.* **28**, 377–387 (2004).
3) H. C. Smith, C. K. Cavanaugh, J. L. Friz, C. S. Thompson, J. A. Saggers, E. L. Michelotti, J. Garcia and C. M. Tice: Synthesis and SAR of *cis*-1-benzoyl-1,2,3,4-tetrahydroquinoline ligands for control of gene expression in ecdysone responsive systems. *Bioorg. Med. Chem. Lett.* **13**, 1943–1946 (2003).
4) S. R. Palli, C. M. Tice, V. M. Margam and A. M. Clark: Biochemical mode of action and differential activity of new ecdysone agonists against mosquitoes and moths. *Arch. Insect Biochem. Physiol.* **58**, 234–242 (2005).
5) E. Becker: Über versuche zur anreicherung und physiologischer charakterisierung des wirkstoffes der puparisierung. *Biol. Zbl.* **61**, 360–388 (1941).
6) Y. Sato, M. Sakai and S. Fujioka: Ecdysone activity of plant-originated molting hormones applied on the body surface of lepidopterous larvae. *Appl. Entomol. Zool.* **3**, 49–51 (1968).
7) M. Ashburner: Effects of juvenile hormone on adult differentiation of *Drosophila melanogaster*. *Nature* **227**, 187–189 (1970).
8) M. J. Fain and L. M. Riddiford: Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.* **149**, 506–521 (1975).
9) C. A. Henrick, W. E. Willy and G. B. Staal: Insect juvenile hormone activity of alkyl (2e,4e)-3,7,11-trimethyl-2,4-dodecadienoates. Vari-

ations in the ester function and in the carbon chain. *J. Agric. Food Chem.* **24**, 207–218 (1976).
10) T. J. Kelly, T. S. Adams, M. B. Schwartz, M. J. Birnbaum, E. C. Rubenstein and R. B. Imberski: Juvenile hormone and ovarian maturation in the Diptera: A review of recent results. *Insect Biochem.* **17**, 1089–1093 (1987).
11) T.-Y. Chen and T.-X. Liu: Susceptibility of immature stages of *Chrysoperla rufilabris* (Neurop., Chrysopidae) to pyriproxyfen, a juvenile hormone analog. *J. Appl. Entomol.* **126**, 125–129 (2002).
12) S. Imoto, T. Nishioka, T. Fujita and M. Nakajima: Hormonal requirements for the larval-pupal ecdysis induced in the cultured integument of *Chilo suppressalis. J. Insect Physiol.* **28**, 1025–1033 (1982).
13) K. Kitahara, T. Nishioka and T. Fujita: Cultured integument of *Chilo suppressalis* as a bioassay system for juvenile hormones. *Agric. Biol. Chem.* **47**, 2841–2847 (1983).
14) C. Y. Clement, D. A. Bradbrook, R. Lafont and L. Dinan: Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. *Insect Biochem. Mol. Biol.* **23**, 187–193 (1993).
15) J. Harmatha and L. Dinan: Biological activity of natural and synthetic ecdysteroids in the B_{II} bioassay. *Arch. Insect Biochem. Physiol.* **35**, 219–225 (1997).
16) H. Oberlander, C. E. Leach and E. Shaaya: Juvenile hormone and juvenile hormone mimics inhibit proliferation in a lepidopteran imaginal disc cell line. *J. Insect Physiol.* **46**, 259–265 (2000).
17) L. Dinan, P. C. Bourne, Y. Meng, S. D. Sarker, R. B. Tolentino and P. Whiting: Assessment of natural products in the *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonist and antagonist activities. *Cell. Mol. Life Sci.* **58**, 321–342 (2001).
18) L. Dinan, P. Bourne, P. Whiting, T. S. Dhadialla and T. H. Hutchinson: Screening of environmental contaminants for ecdysteroid agonist and antagonist activity using the *Drosophila melanogaster* B_{II} cell *in vitro* assay. *Environ. Toxicol. Chem.* **20**, 2038–2046 (2001).
19) G. D. Prestwich, W. S. Eng, M. F. Boehm and C. Wawrzeńczyk: High specific activity tritium- and iodine-labeled JH homologs and analogs for receptor binding studies. *Insect Biochem.* **17**, 1033–1037 (1987).
20) J.-P. Charles, H. Wojtasek, A. J. Lentz, B. A. Thomas, B. C. Bonning, S. R. Palli, A. G. Parker, G. Dorman, B. D. Hammock, G. D. Prestwich and L. M. Riddiford: Purification and reassessment of ligand binding by the recombinant, putative juvenile hormone receptor of the tobacco hornworm, *Manduca sexta. Arch. Insect Biochem. Physiol.* **31**, 371–393 (1996).
21) Y. Nakagawa, C. Minakuchi and T. Ueno: Inhibition of [^{3}H] ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. *Steroids* **65**, 537–542 (2000).
22) Y. Nakagawa, C. Minakuchi, K. Takahashi and T. Ueno: Inhibition of [^{3}H] ponasterone A binding by ecdysone agonists in the intact Kc cell line. *Insect Biochem. Mol. Biol.* **32**, 175–180 (2002).
23) C. Minakuchi, Y. Nakagawa, M. Kamimura and H. Miyagawa: Binding affinity of nonsteroidal ecdysone agonists against the ecdysone receptor complex determines the strength of their molting hormonal activity. *Eur. J. Biochem.* **270**, 4095–4104 (2003).
24) K. Miura, M. Oda, S. Makita and Y. Chinzei: Characterization of the *Drosophila* Methoprene-tolerant gene product: Juvenile hormone binding and ligand-dependent gene regulation. *FEBS J.* **272**, 1169–1178 (2005).
25) T. Ogura, Y. Nakagawa, C. Minakuchi and H. Miyagawa: QSAR for binding affinity of substituted dibenzoylhydrazines to intact Sf-9 cells. *J. Pestic. Sci.* **30**, 1–6 (2005).
26) J.-P. Charles, T. Iwema, V. C. Epa, K. Takaki, J. Rynes and M. Jindra: Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 21128–21133 (2011).
27) M. Jindra, M. Uhlirova, J.-P. Charles, V. Smykal and R. J. Hill: Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. *PLoS Genet.* **11**, e1005394 (2015).
28) L. Bittova, P. Jedlicka, M. Dracinsky, P. Kirubakaran, J. Vondrasek, R. Hanus and M. Jindra: Exquisite ligand stereoselectivity of a *Drosophila* juvenile hormone receptor contrasts with its broad agonist repertoire. *J. Biol. Chem.* **294**, 410–423 (2019).
29) M. R. Koelle, W. S. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas and D. S. Hogness: The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59–77 (1991).
30) T. P. Yao, W. A. Segraves, A. E. Oro, M. McKeown and R. M. Evans: *Drosophila* ultraspiracle modulates ecdysone receptor function *via* heterodimer formation. *Cell* **71**, 63–72 (1992).
31) T. P. Yao, B. M. Forman, Z. Jiang, L. Cherbas, J. D. Chen, M. McKeown, P. Cherbas and R. M. Evans: Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature* **366**, 476–479 (1993).
32) M. Ashok, C. Turner and T. G. Wilson: Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2761–2766 (1998).
33) Y. Nakagawa and V. C. Henrich: Arthropod nuclear receptors and their role in molting. *FEBS J.* **276**, 6128–6157 (2009).
34) T. J. Bernardo and E. B. Dubrovsky: Molecular mechanisms of transcription activation by juvenile hormone: A critical role for bHLH-PAS and nuclear receptor proteins. *Insects* **3**, 324–338 (2012).
35) M. Jindra, X. Bellés and T. Shinoda: Molecular basis of juvenile hormone signaling. *Curr. Opin. Insect Sci.* **11**, 39–46 (2015).
36) J. Alam and J. L. Cook: Reporter genes: Application to the study of mammalian gene transcription. *Anal. Biochem.* **188**, 245–254 (1990).
37) K. V. Wood: Marker proteins for gene expression. *Curr. Opin. Biotechnol.* **6**, 50–58 (1995).
38) C. Suto and D. Ignar: Selection of an optimal reporter gene for cell-based high throughput screening assay. *J. Biomol. Screen.* **2**, 7–9 (1997).
39) D. C. New, D. M. Miller-Martini and Y. H. Wong: Reporter gene assays and their applications to bioassays of natural products. *Phytother. Res.* **17**, 439–448 (2003).
40) T. Jiang, B. Xing and J. Rao: Recent developments of biological reporter technology for detecting gene expression. *Biotechnol. Genet. Eng. Rev.* **25**, 41–76 (2008).
41) L. H. Naylor: Reporter gene technology: The future looks bright. *Biochem. Pharmacol.* **58**, 749–757 (1999).
42) G. Riddihough and H. R. B. Pelham: Activation of the *Drosophila hsp27* promoter by heat shock and by ecdysone involves independent and remote regulatory sequences. *EMBO J.* **5**, 1653–1658 (1986).
43) G. Riddihough and H. R. Pelham: An ecdysone response element in the *Drosophila hsp27* promoter. *EMBO J.* **6**, 3729–3734 (1987).
44) H. E. Thomas, H. G. Stunnenberg and A. F. Stewart: Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* **362**, 471–475 (1993).
45) J. Bai, Y. Uehara and D. J. Montell: Regulation of invasive cell behavior by Taiman, a *Drosophila* protein related to AIB1, a steroid

receptor coactivator amplified in breast cancer. *Cell* **103**, 1047–1058 (2000).

46) L. Cherbas, K. Lee and P. Cherbas: Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev.* **5**, 120–131 (1991).

47) C. Antoniewski, B. Mugat, F. Delbac and J. A. Lepesant: Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster. Mol. Cell. Biol.* **16**, 2977–2986 (1996).

48) D. Martin, S. F. Wang and A. S. Raikhel: The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol. Cell. Endocrinol.* **173**, 75–86 (2001).

49) S.-F. Wang, S. Ayer, W. A. Segraves, D. R. Williams and A. S. Raikhel: Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol.* **20**, 3870–3879 (2000).

50) S. F. Wang, K. Miura, R. J. Miksicek, W. A. Segraves and A. S. Raikhel: DNA binding and transactivation characteristics of the mosquito ecdysone receptor-ultraspiracle complex. *J. Biol. Chem.* **273**, 27531–27540 (1998).

51) S.-F. Wang, C. Li, J. Zhu, K. Miura, R. J. Miksicek and A. S. Raikhel: Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Dev. Biol.* **218**, 99–113 (2000).

52) S. C. Perera, S. Zheng, Q. L. Feng, P. J. Krell, A. Retnakaran and S. R. Palli: Heterodimerization of ecdysone receptor and ultraspiracle on symmetric and asymmetric response elements. *Arch. Insect Biochem. Physiol.* **60**, 55–70 (2005).

53) L. Swevers, L. Cherbas, P. Cherbas and K. Iatrou: *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Mol. Biol.* **26**, 217–221 (1996).

54) J. Zhu, L. Chen, G. Sun and A. S. Raikhel: The competence factor *β*Ftz-F1 potentiates ecdysone receptor activity *via* recruiting a p160/SRC coactivator. *Mol. Cell. Biol.* **26**, 9402–9412 (2006).

55) K. Mikitani: Sensitive, rapid and simple method for evaluation of ecdysteroid agonist activity based on the mode of action of the hormone. *J. Seric. Sci. Jpn.* **64**, 534–539 (1995).

56) K. Mikitani: A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3, 5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide shows apparent insect molting hormone activities at molecular and cellular levels. *Biochem. Biophys. Res. Commun.* **227**, 427–432 (1996).

57) K. Mikitani: Ecdysteroid receptor binding activity and ecdysteroid agonist activity at the level of gene expression are correlated with the activity of dibenzoyl hydrazines in larvae of *Bombyx mori. J. Insect Physiol.* **42**, 937–941 (1996).

58) L. Swevers, L. Kravariti, S. Ciolfi, M. Xenou-Kokoletsi, N. Ragoussis, G. Smagghe, Y. Nakagawa, B. Mazomenos and K. Iatrou: A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. *FASEB J.* **18**, 134–136 (2004).

59) S. T. Suhr, E. B. Gil, M.-C. Senut and F. H. Gage: High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7999–8004 (1998).

60) C. M. Tice, R. E. Hormann, C. S. Thompson, J. L. Friz, C. K. Cavanaugh and J. A. Saggers: Optimization of *α*-acylaminoketone ecdysone agonists for control of gene expression. *Bioorg. Med. Chem. Lett.* **13**, 1883–1886 (2003).

61) M. B. Kumar, D. W. Potter, R. E. Hormann, A. Edwards, C. M. Tice, H. C. Smith, M. A. Dipietro, M. Polley, M. Lawless, P. R. Wolohan, D. R. Kethidi and S. R. Palli: Highly flexible ligand binding pocket of ecdysone receptor: A single amino acid change leads to discrimination between two groups of nonsteroidal ecdysone agonists. *J. Biol. Chem.* **279**, 27211–27218 (2004).

62) S. Lapenna, J. Friz, A. Barlow, S. R. Palli, L. Dinan and R. E. Hormann: Ecdysteroid ligand-receptor selectivity-exploring trends to design orthogonal gene switches. *FEBS J.* **275**, 5785–5809 (2008).

63) S. Lapenna, L. Dinan, J. Friz, A. J. Hopfinger, J. Liu and R. E. Hormann: Semi-synthetic ecdysteroids as gene-switch actuators: Synthesis, structure–activity relationships, and prospective ADME properties. *ChemMedChem* **4**, 55–68 (2009).

64) A. S. Mhashilkar, S. L. Vankayala, C. Liu, F. Kearns, P. Mehrotra, G. Tzertzinis, S. R. Palli, H. L. Woodcock and T. R. Unnasch: Identification of ecdysone hormone receptor agonists as a therapeutic approach for treating filarial infections. *PLoS Negl. Trop. Dis.* **10**, e0004772 (2016).

65) K. D. Wing: RH 5849, a nonsteroidal ecdysone agonist: Effects on a *Drosophila* cell line. *Science* **241**, 467–469 (1988).

66) K. D. Wing, R. A. Slawecki and G. R. Carlson: RH 5849, a nonsteroidal ecdysone agonist: Effects on larval Lepidoptera. *Science* **241**, 470–472 (1988).

67) T. Toya, H. Fukasawa, A. Masui and Y. Endo: Potent and selective partial ecdysone agonist activity of chromafenozide in Sf9 cells. *Biochem. Biophys. Res. Commun.* **292**, 1087–1091 (2002).

68) X. Dai, L. G. Willis, S. R. Palli and D. A. Theilmann: Tight transcriptional regulation of foreign genes in insect cells using an ecdysone receptor-based inducible system. *Protein Expr. Purif.* **42**, 236–245 (2005).

69) H. Mosallanejad, T. Soin, L. Swevers, K. Iatrou, Y. Nakagawa and G. Smagghe: Non-steroidal ecdysteroid agonist chromafenozide: Gene induction activity, cell proliferation inhibition and larvicidal activity. *Pestic. Biochem. Physiol.* **92**, 70–76 (2008).

70) C. E. Wheelock, Y. Nakagawa, T. Harada, N. Oikawa, M. Akamatsu, G. Smagghe, D. Stefanou, K. Iatrou and L. Swevers: High-throughput screening of ecdysone agonists using a reporter gene assay followed by 3-D QSAR analysis of the molting hormonal activity. *Bioorg. Med. Chem.* **14**, 1143–1159 (2006).

71) T. Soin, E. De Geyter, H. Mosallanejad, M. Iga, D. Martin, S. Ozaki, S. Kitsuda, T. Harada, H. Miyagawa, D. Stefanou, G. Kotzia, R. Efrose, V. Labropoulou, D. Geelen, K. Iatrou, Y. Nakagawa, C. R. Janssen, G. Smagghe and L. Swevers: Assessment of species specificity of moulting accelerating compounds in Lepidoptera: Comparison of activity between *Bombyx mori* and *Spodoptera littoralis* by *in vitro* reporter and *in vivo* toxicity assays. *Pest Manag. Sci.* **66**, 526–535 (2010).

72) C. M. Tice, R. E. Hormann, C. S. Thompson, J. L. Friz, C. K. Cavanaugh, E. L. Michelotti, J. Garcia, E. Nicolas and F. Albericio: Synthesis and SAR of *α*-acylaminoketone ligands for control of gene expression. *Bioorg. Med. Chem. Lett.* **13**, 475–478 (2003).

73) J.-M. Wurtz, B. Guillot, J. Fagart, D. Moras, K. Tietjen and M. Schindler: A new model for 20-hydroxyecdysone and dibenzoylhydrazine binding: A homology modeling and docking approach. *Protein Sci.* **9**, 1073–1084 (2000).

74) M. B. Kumar, T. Fujimoto, D. W. Potter, Q. Deng and S. R. Palli: A single point mutation in ecdysone receptor leads to increased ligand specificity: Implications for gene switch applications. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 14710–14715 (2002).

75) I. M. Billas, T. Iwema, J. M. Garnier, A. Mitschler, N. Rochel and D. Moras: Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature* **426**, 91–96 (2003).

76) T. Soin, M. Iga, L. Swevers, P. Rougé, C. R. Janssen and G. Smagghe: Towards Coleoptera-specific high-throughput screening systems for

compounds with ecdysone activity: Development of EcR reporter assays using weevil (*Anthonomus grandis*)-derived cell lines and *in silico* analysis of ligand binding to *A. grandis* EcR ligand-binding pocket. *Insect Biochem. Mol. Biol.* **39**, 523–534 (2009).

77) T. Ogura, Y. Nakagawa, L. Swevers, G. Smagghe and H. Miyagawa: Quantitative evaluation of the molting hormone activity in coleopteran cells established from the colorado potato beetle, *Leptinotarsa decemlineata. Pestic. Biochem. Physiol.* **104**, 1–8 (2012).

78) T. Harada, Y. Nakagawa, T. Ogura, Y. Yamada, T. Ohe and H. Miyagawa: Virtual screening for ligands of the insect molting hormone receptor. *J. Chem. Inf. Model.* **51**, 296–305 (2011).

79) X. Hu, B. Yin, K. Cappelle, L. Swevers, G. Smagghe, X. Yang and L. Zhang: Identification of novel agonists and antagonists of the ecdysone receptor by virtual screening. *J. Mol. Graph. Model.* **81**, 77–85 (2018).

80) T. Soin, L. Swevers, G. Kotzia, K. Iatrou, C. R. Janssen, P. Rouge, T. Harada, Y. Nakagawa and G. Smagghe: Comparison of the activity of non-steroidal ecdysone agonists between dipteran and lepidopteran insects, using cell-based EcR reporter assays. *Pest Manag. Sci.* **66**, 1215–1229 (2010).

81) S. Ito-Harashima and T. Yagi: Unique molecular mechanisms for maintenance and alteration of genetic information in the budding yeast *Saccharomyces cerevisiae. Genes Environ.* **39**, 28 (2017).

82) F. Dela Cruz and P. Mak: *Drosophila* ecdysone receptor functions as a constitutive activator in yeast. *J. Steroid Biochem. Mol. Biol.* **62**, 353–359 (1997).

83) F. E. Dela Cruz, D. R. Kirsch and J. N. Heinrich: Transcriptional activity of *Drosophila melanogaster* ecdysone receptor isoforms and ultraspiracle in *Saccharomyces cerevisiae. J. Mol. Endocrinol.* **24**, 183–191 (2000).

84) H. T. Tran, S. Shaaban, H. B. Askari, P. G. Walfish, A. S. Raikhel and T. R. Butt: Requirement of co-factors for the ligand-mediated activity of the insect ecdysteroid receptor in yeast. *J. Mol. Endocrinol.* **27**, 191–209 (2001).

85) H. T. Tran, H. B. Askari, S. Shaaban, L. Price, S. R. Palli, T. S. Dhadialla, G. R. Carlson and T. R. Butt: Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *Mol. Endocrinol.* **15**, 1140–1153 (2001).

86) M. Lezzi, T. Bergman, V. C. Henrich, M. Vogtli, C. Fromel, M. Grebe, S. Przibilla and M. Spindler-Barth: Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem.* **269**, 3237–3245 (2002).

87) S. Ito-Harashima, M. Matsuura, M. Kawanishi, Y. Nakagawa and T. Yagi: New reporter gene assays for detecting natural and synthetic molting hormone agonists using yeasts expressing ecdysone receptors of various insects. *FEBS Open Bio* **7**, 995–1008 (2017).

88) K. S. Christopherson, M. R. Mark, V. Bajaj and P. J. Godowski: Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6314–6318 (1992).

89) D. No, T. P. Yao and R. M. Evans: Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3346–3351 (1996).

90) E. Oberdörster, M. A. Clay, D. M. Cottam, F. A. Wilmot, J. A. McLachlan and M. J. Milner: Common phytochemicals are ecdysteroid agonists and antagonists: A possible evolutionary link between vertebrate and invertebrate steroid hormones. *J. Steroid Biochem. Mol. Biol.* **77**, 229–238 (2001).

91) S. Ito-Harashima, K. Shiizaki, M. Kawanishi, K. Kakiuchi, K. Onishi, R. Yamaji and T. Yagi: Construction of sensitive reporter assay yeasts for comprehensive detection of ligand activities of human corticosteroid receptors through inactivation of *CWP* and *PDR* genes. *J. Pharmacol. Toxicol. Methods* **74**, 41–52 (2015).

92) S. Ito-Harashima, M. Matano, K. Onishi, T. Nomura, S. Nakajima, S. Ebata, K. Shiizaki, M. Kawanishi and T. Yagi: Construction of reporter gene assays using *CWP* and *PDR* mutant yeasts for enhanced detection of various sex steroids. *Genes Environ.* **42**, 20 (2020).

93) S. R. Palli, M. Z. Kapitskaya, M. B. Kumar and D. E. Cress: Improved ecdysone receptor-based inducible gene regulation system. *Eur. J. Biochem.* **270**, 1308–1315 (2003).

94) A. Goffeau, B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin and S. G. Oliver: Life with 6000 genes. *Science* **274**, 546–567, 563–567 (1996).

95) S. Wang, A. Baumann and T. G. Wilson: *Drosophila melanogaster* Methoprene-tolerant (Met) gene homologs from three mosquito species: Members of PAS transcriptional factor family. *J. Insect Physiol.* **53**, 246–253 (2007).

96) Z.-Q. Li, D.-J. Cheng, L. Wei, P. Zhao, X. Shu, L. Tang, Z.-H. Xiang and Q.-Y. Xia: The silkworm homolog of Methoprene-tolerant (Met) gene reveals sequence conservation but function divergence. *Insect Sci.* **17**, 313–324 (2010).

97) B. Konopova, V. Smykal and M. Jindra: Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS One* **6**, e28728 (2011).

98) A. Baumann, J. Barry, S. Wang, Y. Fujiwara and T. G. Wilson: Paralogous genes involved in juvenile hormone action in *Drosophila melanogaster. Genetics* **185**, 1327–1336 (2010).

99) A. Baumann, Y. Fujiwara and T. G. Wilson: Evolutionary divergence of the paralogs Methoprene tolerant (Met) and germ cell expressed (Gce) within the genus *Drosophila. J. Insect Physiol.* **56**, 1445–1455 (2010).

100) M. A. Abdou, Q. He, D. Wen, O. Zyaan, J. Wang, J. Xu, A. A. Baumann, J. Joseph, T. G. Wilson, S. Li and J. Wang: *Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. *Insect Biochem. Mol. Biol.* **41**, 938–945 (2011).

101) J. Godlewski, S. Wang and T. G. Wilson: Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila. Biochem. Biophys. Res. Commun.* **342**, 1305–1311 (2006).

102) M. Li, E. A. Mead and J. Zhu: Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 638–643 (2011).

103) Z. Zhang, J. Xu, Z. Sheng, Y. Sui and S. R. Palli: Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, Methoprene tolerant. *J. Biol. Chem.* **286**, 8437–8447 (2011).

104) M. Li, P. Liu, J. D. Wiley, R. Ojani, D. R. Bevan, J. Li and J. Zhu: A steroid receptor coactivator acts as the DNA-binding partner of the Methoprene-tolerant protein in regulating juvenile hormone response genes. *Mol. Cell. Endocrinol.* **394**, 47–58 (2014).

105) T. Kayukawa, C. Minakuchi, T. Namiki, T. Togawa, M. Yoshiyama, M. Kamimura, K. Mita, S. Imanishi, M. Kiuchi, Y. Ishikawa and T. Shinoda: Transcriptional regulation of juvenile hormone-mediated induction of *Krüppel homolog 1*, a repressor of insect metamorphosis. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 11729–11734 (2012).

106) T. Kayukawa, K. Tateishi and T. Shinoda: Establishment of a versatile cell line for juvenile hormone signaling analysis in *Tribolium castaneum. Sci. Rep.* **3**, 1570 (2013).

107) Y. Cui, Y. Sui, J. Xu, F. Zhu and S. R. Palli: Juvenile hormone regu-

lates *Aedes aegypti Krüppel homolog 1* through a conserved E box motif. *Insect Biochem. Mol. Biol.* **52**, 23–32 (2014).

108) S. W. Shin, Z. Zou, T. T. Saha and A. S. Raikhel: bHLH-PAS heterodimer of Methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 16576–16581 (2012).

109) H. Miyakawa, K. Toyota, I. Hirakawa, Y. Ogino, S. Miyagawa, S. Oda, N. Tatarazako, T. Miura, J. K. Colbourne and T. Iguchi: A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nat. Commun.* **4**, 1856 (2013).

110) A. W. Olmstead and G. A. Leblanc: Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna. J. Exp. Zool.* **293**, 736–739 (2002).

111) A. W. Olmstead and G. A. LeBlanc: Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna. Environ. Health Perspect.* **111**, 919–924 (2003).

112) N. Tatarazako, S. Oda, H. Watanabe, M. Morita and T. Iguchi: Juvenile hormone agonists affect the occurrence of male *Daphnia. Chemosphere* **53**, 827–833 (2003).

113) H. Miyakawa, M. Watanabe, M. Araki, Y. Ogino, S. Miyagawa and T. Iguchi: Juvenile hormone-independent function of *Krüppel homolog 1* in early development of water flea *Daphnia pulex. Insect Biochem. Mol. Biol.* **93**, 12–18 (2018).

114) D. R. Kethidi, S. C. Perera, S. Zheng, Q.-L. Feng, P. Krell, A. Retnakaran and S. R. Palli: Identification and characterization of a juvenile hormone (JH) response region in the JH esterase gene from the spruce budworm, *Choristoneura fumiferana. J. Biol. Chem.* **279**, 19634–19642 (2004).

115) Q. He, D. Wen, Q. Jia, C. Cui, J. Wang, S. R. Palli and S. Li: Heat shock protein 83 (Hsp83) facilitates Methoprene-tolerant (Met) nuclear import to modulate juvenile hormone signaling. *J. Biol. Chem.* **289**, 27874–27885 (2014).

116) T. Kayukawa and T. Shinoda: Functional characterization of two paralogous JH receptors, Methoprene-tolerant 1 and 2, in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* **50**, 383–391 (2015).

117) T. Yokoi, T. Nabe, C. Ishizuka, K. Hayashi, S. Ito-Harashima, T. Yagi, Y. Nakagawa and H. Miyagawa: Transcription-inducing activity of natural and synthetic juvenile hormone agonists through the *Drosophila* Methoprene-tolerant protein. *Pest Manag. Sci.* **76**, 2316–2323 (2020).

118) T. Tanaka, T. Iguchi and H. Miyakawa: Establishment of a high-sensitivity reporter system in mammalian cells for detecting juvenoids using juvenile hormone receptors of *Daphnia pulex. J. Appl. Toxicol.* **39**, 241–246 (2019).

119) R. Abe, K. Toyota, H. Miyakawa, H. Watanabe, T. Oka, S. Miyagawa, H. Nishide, I. Uchiyama, K. E. Tollefsen, T. Iguchi and N. Tatarazako: Diofenolan induces male offspring production through binding to the juvenile hormone receptor in *Daphnia magna. Aquat. Toxicol.* **159**, 44–51 (2015).

120) H. Miyakawa and T. Iguchi: Comparative luciferase assay for establishing reliable *in vitro* screening system of juvenile hormone agonists. *J. Appl. Toxicol.* **37**, 1082–1090 (2017).

121) T. S. Dhadialla, A. Retnakaran and G. Smagghe: Insect growth- and development-disrupting insecticides. In "Comprehensive Insect Molecular Science, Volume 6: Control," ed. by L. I. Gilbert, I. Kostas, and S. Gill, Pergamon Press, New York, 2005.

122) S.-H. Lee, H.-W. Oh, Y. Fang, S.-B. An, D.-S. Park, H.-H. Song, S.-R. Oh, S.-Y. Kim, S. Kim, N. Kim, A. S. Raikhel, Y. H. Je and S. W. Shin: Identification of plant compounds that disrupt the insect juvenile hormone receptor complex. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1733–1738 (2015).

123) S.-H. Lee, K. B. Ha, D. H. Park, Y. Fang, J. H. Kim, M. G. Park, R. M. Woo, W. J. Kim, I.-K. Park, J. Y. Choi and Y. H. Je: Plant-derived compounds regulate formation of the insect juvenile hormone receptor complex. *Pestic. Biochem. Physiol.* **150**, 27–32 (2018).

124) T. Yokoi, S. Minami, Y. Nakagawa and H. Miyagawa: Structure, activity relationship of imidazothiadiazole analogs for the binding to the ecdysone receptor of insect cells. *Pestic. Biochem. Physiol.* **120**, 40–50 (2015).

125) X. Hu, X. Ma, J. Cui, H. Liu, B. Zhu, J. Xie, P. Liang and L. Zhang: Identification of 1-phenyl-4-cyano-5-aminopyrazoles as novel ecdysone receptor ligands by virtual screening, structural optimization, and biological evaluations. *Chem. Biol. Drug Des.* **97**, 184–195 (2021).

126) B. Jiang, X. Jin, Y. Dong, B. Guo, L. Cui, X. Deng, L. Zhang, Q. Yang, Y. Li, X. Yang and G. Smagghe: Design, synthesis, and biological activity of novel heptacyclic pyrazolamide derivatives: A new candidate of dual-target insect growth regulators. *J. Agric. Food Chem.* **68**, 6347–6354 (2020).

127) W. Birru, R. T. Fernley, L. D. Graham, J. Grusovin, R. J. Hill, A. Hofmann, L. Howell, P. J. James, K. E. Jarvis, W. M. Johnson, D. A. Jones, C. Leitner, A. J. Liepa, G. O. Lovrecz, L. Lu, R. H. Nearn, B. J. O'Driscoll, T. Phan, M. Pollard, K. A. Turner and D. A. Winkler: Synthesis, binding and bioactivity of γ-methylene γ-lactam ecdysone receptor ligands: Advantages of QSAR models for flexible receptors. *Bioorg. Med. Chem.* **18**, 5647–5660 (2010).

128) R. E. Hormann, O. Chortyk and D. P. Le: Oxadiazoline ligands for modulating the expression of exogenous genes *via* an ecdysone receptor complex. US 8895306 B2 (2014).

129) G. Holmwood and M. Schindler: Protein structure based rational design of ecdysone agonists. *Bioorg. Med. Chem.* **17**, 4064–4070 (2009).

130) P. Sharma, S. Thakur and P. Awasthi: *In silico* and bio assay of juvenile hormone analogs as an insect growth regulator against *Galleria mellonella* (wax moth), part I. *J. Biomol. Struct. Dyn.* **34**, 1061–1078 (2016).

131) P. Ramaseshadri, R. Farkaš and S. R. Palli: Recent progress in juvenile hormone analogs (JHA) research. In "Advances in insect physiology," ed. by T. S. Dhadialla, Academic Press, pp. 353–436, 2012.

132) C. A. Henrick: Methoprene. *J. Am. Mosq. Control Assoc.* **23**(Suppl), 225–239 (2007).

133) K. Sláma and C. M. Williams: Juvenile hormone activity for the bug *Pyrrhocoris apterus. Proc. Natl. Acad. Sci. U.S.A.* **54**, 411–414 (1965).

134) L. Dinan, P. Whiting, J.-P. Girault, R. Lafont, S. T. Dhadialla, E. D. Cress, B. Mugat, C. Aantoniewski and J.-A. Lepesant: Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. *Biochem. J.* **327**, 643–650 (1997).

135) M. J. Zotti, E. De Geyter, L. Swevers, A. S. K. Braz, L. P. B. Scott, P. Rougé, J. Coll, A. D. Grutzmacher, E. J. Lenardão and G. Smagghe: A cell-based reporter assay for screening for EcR agonist/antagonist activity of natural ecdysteroids in Lepidoptera (Bm5) and Diptera (S) cell cultures, followed by modeling of ecdysteroid-EcR interactions and normal mode analysis. *Pestic. Biochem. Physiol.* **107**, 309–320 (2013).

136) D. L. Bain, A. F. Heneghan, K. D. Connaghan-Jones and M. T. Miura: Nuclear receptor structure: Implications for function. *Annu. Rev. Physiol.* **69**, 201–220 (2007).

137) J. M. Olefsky: Nuclear receptor minireview series. *J. Biol. Chem.* **276**, 36863–36864 (2001).

138) A. Aranda and A. Pascual: Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**, 1269–1304 (2001).

139) K. D. Baker, J. T. Warren, C. S. Thummel, L. I. Gilbert and D. J. Mangelsdorf: Transcriptional activation of the *Drosophila* ecdysone receptor by insect and plant ecdysteroids. *Insect Biochem. Mol. Biol.* **30**, 1037–1043 (2000).

140) G.-P. Song, D.-K. Hu, H. Tian, Y.-S. Li, Y.-S. Cao, H.-W. Jin and Z.-N. Cui: Synthesis and larvicidal activity of novel thenoylhydrazide derivatives. *Sci. Rep.* **6**, 22977 (2016).