Chapter 6
Tyrosine Metabolism for Insect Cuticle Pigmentation and Sclerotization

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Abstract Pigmentation or body color patterns in insects quite often differ not only between species but also in different stages of development and in different body regions of a single species. Body coloration plays physiologically and ecologically important roles as for instance in species recognition and communication, courtship/mate selection, mimicry, crypsis, warning, prey-predator/parasite interactions, and resistance to temperature, desiccation and absorbs or reflects harmful ultraviolet radiation. Many kinds of pigment molecules and structural colors contribute to the diversity of body coloration in insects. Recent studies have elucidated some of the genetic and molecular biological mechanisms underlying pigment biosynthesis. This chapter focuses on the pigments derived from the amino acid tyrosine. The tyrosine-mediated cuticle tanning pathway is responsible for production of melanins and other pigments derived from 3,4-dihydroxyphenylalanine (DOPA) and dopamine as well as from *N*-acyldopamines. The *N*-acylated dopamines, in addition, are oxidized by the phenoloxidase laccase 2 to form quinones and quinone methides, which then undergo cross-linking reactions with cuticular proteins (CPs) for cuticle sclerotization. We review the regulation and functional importance and also the diversity of the genes involved in this pathway. The unique localization and cross-linking of specific CPs for morphology and ultrastructure of the exoskeleton are also discussed.

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6.1 Introduction

6.1.1 Insect Cuticle Composition and Morphology

The insect exoskeleton or cuticle is composed of multiple functional layers including an outermost lipophilic waterproofing envelope, a protein-rich epicuticle and a chitin/protein-rich procuticle that makes up the major portion (see Chap. 7 in this book for details about insect hydrocarbons; Locke 2001; Moussian et al. 2006; Moussian 2010). It plays critical roles in protecting insects from various physical and environmental stresses and from pathogenic challenges. Two different structural biopolymers, cuticular proteins (CPs) and chitin (see Chap. 2 in this book for details about chitin metabolism), are the major components of the exo- and endocuticular layers that comprise the procuticle (Willis 2010). Other components include pigments, catechols, mineral salts, lipids and water. The primary focus of this review is the pigments found in insects that are derived from the amino acid tyrosine, which include melanins and papiliochromes with the former being the predominant class (Wittkopp and Beldade 2009; Shamim et al. 2014). Melanogenesis is a complex multistep production of high molecular weight melanins via hydroxylation, oxidation and polymerization of the oxidized metabolites (Singh et al. 2013). Although melanin-type pigments commonly occur in insect cuticles, their isolation and partial characterization have been carried out in only a few species (Hackman 1974, 1984; Hori et al. 1984; Kayser 1985). No definitive molecular structure has yet been delineated for these heterogeneous polymers, but they appear to be combinations of phenolic, indolic, pyrrolic and aliphatic structures that may interact covalently and noncovalently with macromolecular components such as cuticular proteins and chitin (Schaefer et al. 1987; Duff et al. 1988; Solano 2014; Chatterjee et al. 2015).

There are several other kinds of pigments such as pterins, ommochromes, antraquinones, aphins (polycyclic quinones), tertapyrroles, carotenoids and flavonoids/anthocyanins present in insect tissues, many of which have been described in earlier reviews (Cromartie 1959; Fuzeau-Braesch 1972; Takeuchi et al. 2005; Shamim et al. 2014). Ommochromes, for example, are one of the major pigments that have been found in eyes, eggs and body walls of insects. In ommochrome biosynthesis, tryptophan is converted to 3-hydroxykynurenine, which is then incorporated into pigment granules by ABC transporters (Tearle et al. 1989; Pepling and Mount 1990). In addition, another transporter, which is a member of a major facilitator superfamily, incorporates other precursor(s) into granules (Osanai-Futahashi et al. 2012b) where the pigments are synthesized via oxidative polymerization. Aphines synthesized from a presumed polyketide precursor have only been found in aphids and contribute to a variety of body colors during development and/or different species. Tsuchida et al. (2010) demonstrated that infection with an aphid endosymbiont of the genus *Rickettsiella* increases the amounts of blue-green aphins in the pea aphid, *Acyrthosiphon pisum*, resulting in a change in their body color from reddish to greenish. This body color change caused by endosymbiosis is likely to influence prey-predator/parasite interactions and natural populations of the aphid. Pterins

synthesized from guanosine triphosphate are also widely distributed in eyes, bodies and wings of insects. In addition, tetrahydrobiopterin (BH_4) serves as a cofactor for enzymes such as phenylalanine hydroxylase and tyrosine hydroxylase (TH) in tyrosine metabolism-associated cuticle pigmentation and sclerotization (Futahashi et al. 2010). For example, 6-pyruvoyl-tetrahydropterin synthase (PTS, Purple) is involved in the BH_4 biosynthesis, and a mutation in *BmPTS* gene is responsible for colorless cuticle of the albino (*al*) mutant of the silkworm, *Bombyx mori* (Fujii et al. 2013).

Insects and mammals possess fine-tuned systems of enzymes to meet their specific demands for tyrosine metabolites. In addition, more closely related enzymes involved in tyrosine metabolism appear to have emerged in many insect species (Vavricka et al. 2014). The metabolism of tyrosine plays a major role in not only the darkening of insect cuticle but also in its hardening or sclerotization as well as in innate immune responses to microbial pathogens. Coloration can vary from colorless to yellow to tan to orange to brown to black depending on the amounts and types of melanin-like pigments incorporated. The degree of sclerotization can vary from soft and flexible to hard and stiff, much of which is determined by the number of chitin-protein laminae and also the number of structural proteins' cross-links derived from tyrosine metabolism (Yang et al. 2014). The chemistry underlying insect pigmentation is rather complex. However, substantial progress has been made in recent years in understanding how tyrosine metabolism contributes to that process (Sugumaran 2009; Shamim et al. 2014).

CPs and chitin are the major components of the exo- and endocuticular layers that comprise the procuticle. The former layer is generally formed before molting, whereas the latter is mainly deposited after completion of the molting process. Transmission electron microscopic (TEM) analysis, for example, revealed that the embryonic cuticle of the fruit fly, *Drosophila melanogaster*, as well as dorsal larval body wall cuticle from the red flour beetle, *Tribolium castaneum*, are composed of an envelope, epicuticle and procuticle, the latter consisting of various numbers of horizontally oriented chitin-protein laminae parallel to the epidermal cell's apical plasma membrane (Fig. 6.1a; Moussian et al. 2006; Moussian 2010). These morphologically distinct layers are also evident in cuticle of elytra (modified forewings; Chen et al. 2015a, b) dissected from *T. castaneum* pharate adults, which become harder and darker shortly after eclosion. In the case of elytral dorsal cuticle, unlike the relatively soft and flexible elytral ventral and larval dorsal body wall cuticles, there are numerous vertically oriented columnar structures denoted as pore canal fibers (PCFs), which extend directly from the "apical plasma membrane protrusions" (APMP) of the underlying epidermal cells and penetrate a large number of horizontal laminae, reaching all the way to the epicuticle (Fig. 6.1b; Noh et al. 2014). Not only the horizontal laminae but also the vertical PCFs are likely composed of chitin as those structures bind to wheat germ agglutinin (Noh et al. 2015b). In *T. castaneum* adults, other regions with rigid cuticle such as the thoracic body wall and leg exhibit an ultrastructure very similar to that of the elytron's dorsal cuticle. On the other hand, there are fewer horizontal laminae and no vertical pore canals with PCFs in anatomical regions covered with soft, flexible and less pigmented cuticles such as those found on the dorsal abdomen, ventral elytron and

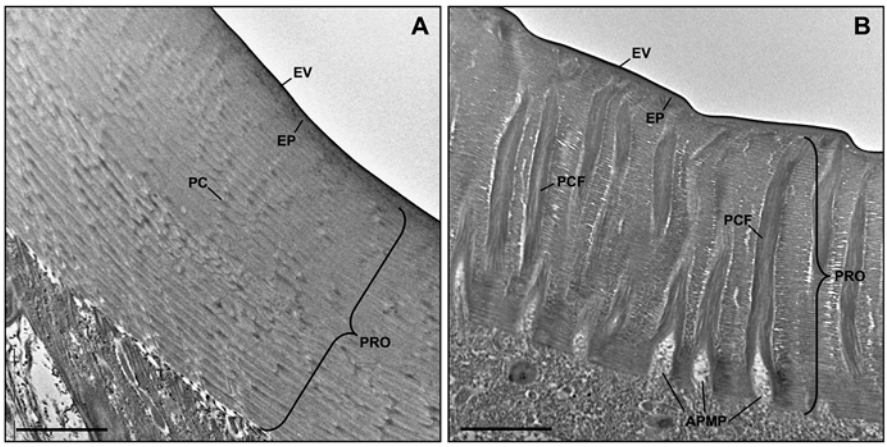

Fig. 6.1 Ultrastructure of larval (a) and adult body wall (b) cuticles in *T. castaneum*. Both larval and adult body cuticles are composed of distinct layers including the envelope, epicuticle and procuticle. The procuticle consists of a number of horizontal chitinous laminae in larval and adult body wall cuticles. In addition, there are numerous pore canals running transverse to the laminae, as well as to the apical plasma membrane. The canals extend from the apical plasma membrane to the epicuticle region and contain a core of pore canal fibers in adult body cuticle. Ultrastructure of adult body wall is similar to those of elytra and leg cuticles in *T. castaneum*, which are relatively hard cuticles (Noh et al. 2014). *EV* envelope, *EP* epicuticle, *PRO* procuticle, *PC* pore canal, *PCF* pore canal fiber, *APMP* apical plasma membrane protrusion. Scale bar = 2 μm

hindwing (Noh et al. 2014). Similar vertical fibrillar structures or vertical fibrils have been observed not only in other insect species (Locke 1961; Delachambre 1971; Wigglesworth 1985) but also in the exoskeletons of crustaceans after removal of minerals and some proteins, including *Homarus americanus* (American lobster), *Callinectes sapidus* (Atlantic blue crab) and *Tylos europaeus* (sand-burrowing isopod) (see Chap. 5 in this book for details about exoskeletons of crustaceans; Raabe et al. 2006; Cheng et al. 2008; Seidl et al. 2011), suggesting that this unique architecture and arrangement of numerous laminae and PCFs contribute to the physical strength of rigid cuticle in arthropods. Pigments are found in cuticle and epidermis and little or no information is available as to whether they contribute to the mechanical properties of the exoskeleton.

6.1.2 Proposed Tyrosine-Mediated Cuticle Tanning Pathway

Despite a rather limited composition, cuticle has remarkably diverse mechanical properties, ranging from soft and flexible to hard and rigid. An insect must periodically replace its old cuticle with a new one by undergoing ecdysis because the mature cuticle is too restrictive to allow for continuous growth during development. Immediately after molting, the cuticle is soft and pale, but it shortly becomes

hardened (sclerotized) and often darker (pigmented) over a period of several hours or days. This vital process together with dehydration occurs during each stage of development (Kramer et al. 2001; Andersen 2005; Arakane et al. 2008; Lomakin et al. 2011).

Tanning (pigmentation and sclerotization) is a complex and important physiological event not only in cuticle formation (Andersen 2010) but also in wound healing, encapsulation during a defensive response to infection of parasites, and hardening of the egg chorion (see Chap. 9 in this book for details about chorion hardening; Li 1994; Sugumaran 2002). With tyrosine as the initial substrate, the cuticle tanning reactions include hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and decarboxylation of DOPA to dopamine. For melanization, furthermore, oxidation of DOPA and dopamine to DOPA-quinone and dopaminequinone, conversion of these quinones to dihydroxyindole (DHI) and/or 5,6-dihydroxyindole-2-carboxylic acid (DHICA), oxidation of DHI and DHICA to DHI-chrome and DHICA-chrome (melanochromes) and finally polymerization of melanochromes to form melanins (Fig. 6.2; Arakane et al. 2009; Simon et al. 2009). For pigmentation involving acylated quinones, the reactions include *N*-acetylation of dopamine to *N*-acetyldopamine (NADA) or *N*-β-alanylation to *N*-β-alanyldopamine (NBAD), oxidation of NADA and NBAD to NADA-quinone and NBAD-quinone and their polymerization to form their corresponding pigments. These quinones, in addition, undergo isomerization to quinone methides and cross-linking reactions with CP side chains (most likely histidyl residues) for cuticle sclerotization (Fig. 6.2; Kramer et al. 2001).

Recent studies have indicated that the following enzymes are involved in the catalysis of cuticle tanning reactions: tyrosine hydroxylase (TH; Pale) converts tyrosine to DOPA; DOPA decarboxylase (DDC) converts DOPA to dopamine; dopachrome conversion enzyme (DCE, Yellow) accelerates the conversion of dopachrome to DHI; arylalkylamine *N*-acetyltransferase (NAT) converts dopamine to NADA; aspartate 1-decarboxylase (ADC) decarboxylates L-aspartic acid to β-alanine for production of NBAD; NBAD synthase (Ebony) produces NBAD and laccase 2 catalyzes the catecholic oxidation reactions in the tanning pathway (Table 6.1 and see Sect. 6.2 for details).

6.2 Functions of Key Enzymes/Proteins Involved in Tyrosine-Mediated Cuticle Tanning

6.2.1 Tyrosine Hydroxylase (TH)

The first step in the cuticle tanning pathway is the hydroxylation of tyrosine to produce DOPA (Fig. 6.2). There are two enzymes that can catalyze this hydroxylation reaction, tyrosinase (phenoloxidase, PO) and tyrosine hydroxylase (TH). Although the former enzyme has been detected in insect cuticles and is multifunctional,

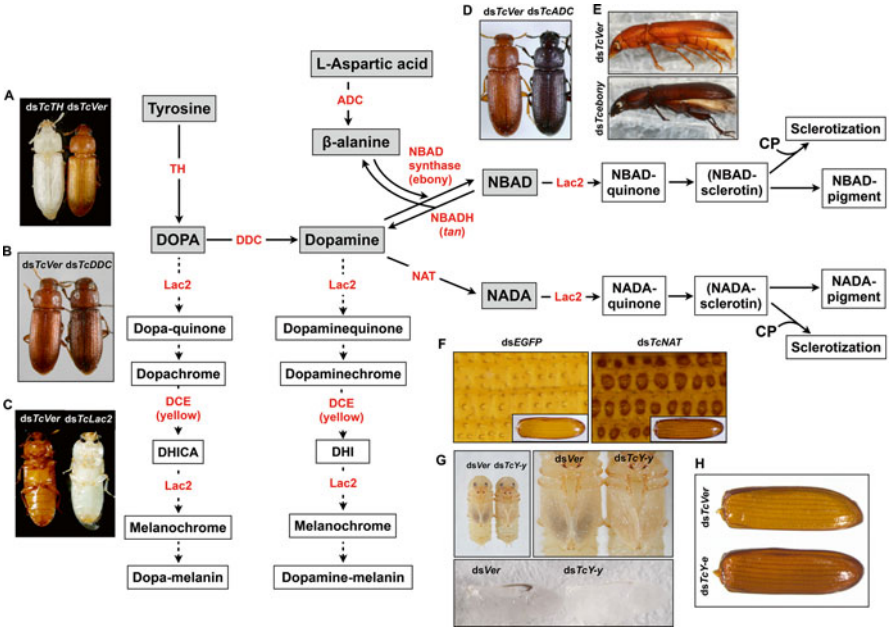

Fig. 6.2 Proposed cuticle tanning pathway in *T. castaneum*. DOPA, 3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenethylamine; NADA, *N*-acetyldopamine; NBAD, *N*-β-alanyldopamine; TH, tyrosine hydroxylase; DDC, DOPA decarboxylase; NAT, *N*-acetyltransferase; NBAD synthase (Ebony), *N*-β-alanyldopamine synthase; ADC, aspartate 1-decarboxylase; NBADH (Tan), *N*-β-alanyldopamine hydrolase; Lac2, laccase 2; DCE (Yellow), dopachrome conversion enzyme; CP, cuticle proteins. The *broken* and *solid lines* represent melanin synthesis and quinone tanning pathways, respectively. Key enzymes are indicated in red letters and body color changes are shown after RNAi for *TcTH* (**a**), *TcDDC* (**b**), *TcLac2* (**c**), *TcADC* (**d**), *Tcebony* (**e**), *TcNAT* (**f**), *TcY-y* (**g**) and *TcY-e* (**h**)

oxidizing *ortho*-diphenols as well as hydroxylating monophenols such as tyrosine (Andersen 2005; Arakane et al. 2005; Kanost and Gorman 2008), it likely plays only a role in immune-related melanization and not in cuticle tanning as suggested by several studies (Barrett 1991; Marmaras et al. 1996; Ashida and Brey 1997; Asano and Ashida 2001; Christensen et al. 2005; Kanost and Gorman 2008). In line with this notion, double-stranded RNA (dsRNA)-mediated loss of function of tyrosinase in *T. castaneum*, for instance, had no effect on larval, pupal and adult cuticle tanning (Arakane et al. 2005).

Like mammalian TH, insect TH is a pterin (BH_4)-dependent oxygenase (Liu et al. 2010; Fujii et al. 2013) that catalyzes hydroxylation of tyrosine as a homotetramer. Its activity is regulated by phosphorylation of a serine residue catalyzed by a cAMP-dependent protein kinase (Vie et al. 1999). In *D. melanogaster*, there are two alternatively spliced isoforms of *DmTH* (*pale*): the longer is expressed in the epidermis and the shorter, which lacks a highly acidic region in the N-terminus, specifically is expressed in the central nervous system (Birman et al. 1994; Friggi-Grelin

Table 6.1 Cuticle tanning-related genes and loss of function phenotypes in insects

Enzyme	Species	Accession no.	Loss of function	Phenotype	References
Tyrosine hydroxylase (TH)	*Tribolium castaneum*	EF592178	RNAi	Colorless and soft cuticle	Gorman and Arakane (2010)
	Drosophila melanogaster	AAF50648	Mutant (*pale*)	Unpigmented embryos/ pale cuticle	Budnik and White (1987), Neckameyer and White (1993), and True et al. (1999)
	Manduca sexta	EF592177	–	–	Gorman et al. (2007)
	Papilio xuthus	AB178006	Chemical inhibition	Suppression of larval pigmentation	Futahashi and Fujiwara (2005)
	Pseudaletia separata	AB274834	Chemical inhibition	Diminished black pigmentation of larval stripes	Ninomiya et al. (2006)
	Bombyx mori	AB439286	Mutant (sch)/RNAi	Light reddish-brown neonate larval body color/delay pupal pigmentation	Liu et al. (2010) and Lee et al. (2015)
	Spodoptera exigua	JF795467 JF795468	Mutant (overexpression)	Dark pigmented pupal cuticle	Liu et al. (2015)
	Oncopeltus fasciatus	KM247780	RNAi	Loss of black pigmentation in adult cuticle	Liu et al. (2014)

(continued)

Table 6.1 (continued)

Enzyme	Species	Accession no.	Loss of function	Phenotype	References
Dopa decarboxylase (DDC)	*Tribolium castaneum*	EU019710	RNAi	Slightly darker adult cuticle	Arakane et al. (2010)
	Drosophila melanogaster	AAF53764	Mutant	Albino adult bristle and cuticle	True et al. (1999)
	Pseudaletia separata	AB072300	Chemical inhibition	Diminished black pigmentation of larval stripes	Ninomiya et al. (2006)
	Papilio xuthus	AB178007	Chemical inhibition	Suppression of larval pigmentation	Futahashi and Fujiwara (2005)
	Bombyx mori	AF372836	RNAi	Fail larval to pupal molt	Wang et al. (2013)
	Papilio glanucus	AF036963	Mutant (melanic female)	Yellow proximal area of adult wing alters black	Koch et al. (1998)
	Spodoptera exigua	SEU71404	Mutant (overexpression)	Dark pigmented pupal cuticle	Liu et al. (2015)
	Oncopeltus fasciatus	KM247781	RNAi	Reduction of adult black pigmentation	Liu et al. (2014)
Aspartate 1-decarboxylase (ADC)	*Tribolium castaneum*	EU019709	RNAi/ mutant (*black* and b^{chr}/b^{ST})	Black body pigmentation in larva, pupa and adult	Kramer et al. (1984) and Arakane et al. (2010)
	Drosophila melanogaster	NM_057440	mutant (*black*)	Black/dark body pigmentation	Phillips et al. (1993, 2005)
	Bombyx mori	KM523624	mutant (*bp*)	Black pupal cuticle	Dai et al. (2015)

Arylalkylamine *N*-acetyltransferase (NAT)	*Tribolium castaneum*	FJ647798	RNAi	Dark pigmented adult cuticle	Tomoyasu et al. (2009)
	Bombyx mori	DQ256382	RNAi/mutant (*mln*)	Darker body pigmentation	Dai et al. (2010), Zhan et al. (2010), and Qiao et al. (2012)
			overexpression	Dark coloration of neonatal larvae, adult antenna and larval tracheae alters light brawn	Osanai-Futahashi et al. (2012)
NBAD synthase (Ebony)	*Tribolium castaneum*	FJ647797	RNAi	Dark pigmented adult cuticle	Tomoyasu et al. (2009)
	Drosophila melanogaster	AAF55870	mutant	Dark pigmented adult thorax and abdomen	Wittkopp et al. (2002a)
	Papilio xuthus	AB195255	–	–	Futahashi and Fujiwara (2005)
	Bombyx mori	AB439000	Mutant (*sooty*)	Dark body pigmentation in larva and pupa	Futahashi et al. (2008a, b)
	Papilio glanucus	–	Mutant (melanic female)	Black melanin replaces yellow background in wing	Koch et al. (2000)
	Papilio polytes	AB525746	–	–	Nishikawa et al. (2013)

(continued)

Table 6.1 (continued)

Enzyme	Species	Accession no.	Loss of function	Phenotype	References
NBAD hydrolase (Tan)	*Drosophila melanogaster*	NM_132315	Mutant	Less pigmented adult thorax and abdomen	True et al. (2005), Jeong et al. (2008), and Aust et al. (2010)
	Bombyx mori	AB499125	Mutant (*ro*)	Larval black marking alters light brown	Futahashi et al. (2010)
	Papilio xuthus	AB499122	–	–	Futahashi et al. (2010)
Laccase 2 (Lac2)	*Tribolium castaneum*	AY884061	RNAi	Colorless and soft cuticle, lethal	Arakane et al. (2005)
	Monochamus alternatus	EU093075	RNAi	Colorless and soft cuticle, lethal	Niu et al. (2008)
	Riptortus pedtris	AB586067	RNAi	Colorless and soft cuticle, lethal	Futahashi et al. (2011)
	Nysius plebeius	AB586069	RNAi	Less pigmentation	Futahashi et al. (2011)
	Megacopta punctatissima	AB586068	RNAi	Less pigmentation	Futahashi et al. (2011)
	Papilio xuthus	AB499123	–	–	Futahashi et al. (2010)
	Oncopeltus fasciatus	KM247782	RNAi	Reduction of adult black pigmentation	Liu et al. (2014)
	Nilaparvata lugens	KR086787	RNAi	Colorless and soft cuticle, lethal	Ye et al. (2015)

Yellow-y (Y-y)	*Tribolium castaneum*	GU111770	RNAi	Not critical for body coloration except for black pigmented pterostigma of the hindwing	Arakane et al. (2010)
	Drosophila melanogaster	NM_057444	mutant	Light brown/yellowish cuticle	Wittkopp et al. (2002a)
			overexpression	Black adult wing cuticle	Riedel et al. (2011)
	Bombyx mori	AB438999	mutant (*ch*)	Lighter pigmented larval cuticle	Futahashi et al. (2008a, b)
	Papilio xuthus	AB195254	–	–	Futahashi and Fujiwara (2007)
Yellow-e (Y-e)	*Tribolium castaneum*	GU111765	RNAi	Dark body color in adult body cuticle and vein of hindwing	Noh et al. (2015a)
	Bombyx mori	AB489224	mutant (*bts*)	White larval head and anal plates alters reddish brown	Ito et al. (2010)

et al. 2003). Similarly, long epidermal and short brain/neural isoforms of the *TH* transcript were identified in the oriental armyworm, *Pseudaletia separata* (Ninomiya and Hayakawa 2007).

Several studies have implicated a functional importance of TH in insect cuticle pigmentation. Mutation in the *DmTH* gene (*pale*) results in lethal unpigmented embryos that fail to develop into larvae, resulting in no hatching from eggs (Budnik and White 1987; Neckameyer and White 1993). Because TH is also important in the nervous system, it is possible that the embryos die before becoming fully developed because of the lack of DmTH function in this system. This embryonic lethality was rescued by expression of the epidermal form of DmTH (Friggi-Grelin et al. 2003). True et al. (1999) demonstrated by using mosaic analysis that the lack of DmTH function resulted in an adult albino cuticle phenotype, and ectopic DmTH expression caused ectopic cuticle pigmentation. In lepidopteran species such as the Asian swallowtail, *Papilio xuthus* and *P. separata*, *TH* mRNA and/or protein were detected in the epidermal cells underlying the darkly marked larval cuticle (Futahashi and Fujiwara 2005; Ninomiya and Hayakawa 2007). In the tobacco hornworm, *Manduca sexta*, the amount of TH protein in the integument of pharate pupal segments is correlated with the degree of cuticle pigmentation (Gorman et al. 2007). Treatment with a TH inhibitor such as 3-iodotyrosine inhibited pigment formation in *P. xuthus* larvae and *B. mori* adults (Futahashi and Fujiwara 2005; Lee et al. 2015) and also rescued black pupae produced by overexpressed *TH* and *DDC* genes in a pupal melanic mutation strain of the beet armyworm, *Spodoptera exigua* (Liu et al. 2015). In addition, RNAi for *BmTH* caused a delay in pupal cuticle pigmentation of *B. mori* (Lee et al. 2015), and a reduction of *BmTH* transcripts was responsible for the sex-linked chocolate (*sch*) mutant, which exhibits a light reddish-brown neonatal larval body color compared with the black color of that of the wild-type strain (Liu et al. 2010). There is differential regulation of TH during cuticular melanization and innate immunity in *B. mori* (Lee et al. 2015). BmTH is expressed in the epidermis during development for the purpose of pupal cuticle melanization and pigmentation in adults, and in the fat body during infection for antimicrobial activity. In the large milkweed bug, *Oncopeltus fasciatus*, RNAi for the *OfTH* gene resulted in a complete absence of black pigmentation in adult cuticles on the head, thorax, abdomen and wings (Liu et al. 2014). Similarly, in *T. castaneum*, injection of dsRNA for *TcTH* diminished the brown pigmentation in the pupal cuticle of the abdominal segments, urogomphi, bristles and gin traps as well as in the adult cuticle of the mandibles and legs, which was visible underneath the pupal cuticle, and also the dark pigmentation observed in the hindwings. The cuticle of phypomorphic ds*TcTH*-treated adults is pale (Fig. 6.2a; Gorman and Arakane 2010). Furthermore, the cuticles of both hard and dark dorsal thorax and hard and colorless eye of ds*TcTH*-treated adults are soft and flexible, indicating that TH is required for not only cuticle pigmentation but also for sclerotization in some species.

6.2.2 *Dopa Decarboxylase (DDC)*

Dopamine is a catecholamine involved in nervous systems of mammals and insects as a neurotransmitter, neuromodulator and neurohormone (Nassel 1996; Osborne 1996; Neckameyer and Leal 2002; Han et al. 2010). In insects, dopamine is also important for egg-shell hardening and the immune response as well as cuticle melanization (Hopkins et al. 1984; Nappi et al. 1992; Huang et al. 2005; Davis et al. 2008; Paskewitz and Andreev 2008; Sideri et al. 2008). In the cuticle tanning pathway, DOPA decarboxylase (DDC), which is a pyridoxal-5-phosphate (PLP)-dependent enzyme, catalyzes the decarboxylation of DOPA to yield dopamine, a major precursor in melanin/pigment production and sclerotization mediated by protein cross-linking (Hopkins et al. 1984; Hiruma et al. 1985; Kramer and Hopkins 1987; Andersen 2005; Hopkins and Kramer 1992; Riddiford et al. 2003).

The functional importance of DDC in cuticle coloration has been well studied in several lepidopteran species, which exhibit a high degree of variation in pigmentation. Like the *TH* gene, *DDC* is highly expressed in epidermal cells underlying the presumptive black markings (e.g. eyespots, V-shaped and band-markings) of larval cuticles of different swallowtail butterflies including *P. xuthus*, the common yellow swallowtail, *Papilio machaon* and the common Mormon, *Papilio polytes* (Shirataki et al. 2010). Furthermore, chemical inhibitors of DDC such as *m*-hydroxybenzylhydrazine (HBHZ) completely inhibited pigment formation of *P. xuthus* (Futahashi and Fujiwara 2005). In the eastern tiger swallowtail, *Papilio glanucus*, *DDC* expression and enzyme activity regulate the color pattern of the adult wings (Koch et al. 1998). *DDC* mRNA and activity are detected early in the presumptive yellow regions of the wings and later in the presumptive black patterns. In the melanic female of this species, early DDC activity in the central yellow region of the wing is much lower than that of wild-type females, which is later melanized concomitant with increased DDC activity. Ninomiya et al. (2006) demonstrated that DDC expression is required for the black strips in the dorsal cuticle of last instar larvae of *P. separata*. *DDC* mRNA and protein are detected in the epidermal cells underneath the black stripe, but not below the white stripe. HBHZ treatment caused the complete loss of DDC activity, a low level of dopamine, an abnormally high level of DOPA and diminished black pigmentation of the strips in the larval cuticle, indicating that DDC activity and its product dopamine are critical for melanin deposition in the black strips.

In D. melanogaster, the *DmDDC* gene has two alternatively spliced transcripts, of which one is expressed in the epidermis and the other in the central nervous system (Hodgetts and O'Keefe 2006). Tissue-specific expression of alternatively spliced DDC isoforms has not been reported in other species. The crystal structure and site-directed mutagenesis analysis of DmDDC indicate that T82 is involved in substrate binding and H192 is essential for both a substrate interaction and cofactor binding (Han et al. 2010). These amino acid residues are highly conserved among insect DDCs. Like that observed for DmTH, patches of epithelial cells deficient in DmDDC activity produce albino adult bristles and cuticle (True et al. 1999).

Similarly, loss of function of *DDC* by RNAi causes a reduction in black pigmentation of *O. fasciatus* adult cuticle (Liu et al. 2014). In *T. castaneum*, the level of DOPA increases approximately 5-fold in ds*TcDDC*-treated pharate adults (Arakane et al. 2009). However, the initial cuticle pigmentation of the resulting adults is substantially delayed, suggesting that, unlike dopamine, DOPA does not appear to be utilized to a great extent for DOPA quinone-associated melanin synthesis, probably because DOPA is a poor substrate for laccase 2 (Arakane et al. 2009). The body color of the TcDDC-deficient mature adults is slightly darker than that of control animals (Fig. 6.2b). This phenotype may be due to a small amount of DOPA-melanin accumulation relative to NBAD- and/or NADA-derived pigments in the adult cuticle. All of these results indicate that DDC activity is required for providing dopamine as a major precursor for melanin synthesis and also quinone-based tanning in insect cuticle.

6.2.3 Aspartate 1-Decarboxylase (ADC)

β-Alanine is involved in critical physiological events in insects. In cuticle tanning, it is conjugated with dopamine via the action of NBAD synthase (*ebony*; see Sect. 6.2.5) to produce NBAD, which is one of the major catechols serving as a cuticle tanning precursor (Hopkins et al. 1984; Kramer et al. 1984; Andersen 2007, 2010). β-Alanine is produced by decarboxylation of the α-COOH of aspartic acid, and aspartate 1-decarboxylase (ADC) catalyzes this reaction. Like DDC, ADC is a member of the pyridoxal 5-phosphate-dependent amino acid decarboxylase family, in which all members possess a conserved decarboxylase domain containing a pyridoxal phosphate-binding domain motif (Ser-X-X-Lys). Phylogenetic analysis revealed that ADCs are closely related with glutamate decarboxylases (GDCs) and distantly related with other decarboxylase family members including histidine, tyrosine and DOPA decarboxylases (Fig. 6.3; Arakane et al. 2009). Although ADCs and GDCs show a high overall amino acid sequence identity/similarity, they exhibit a rather rigorous substrate specificity probably due to small differences in the enzymes' active sites. Richardson et al. (2010) demonstrated that the recombinant ADC protein from the yellow fever mosquito, *Aedes aegypti*, produces β-alanine by decarboxylation of aspartic acid, but it is inactive toward glutamic acid. On the other hand, the recombinant GDC from the malaria mosquito, *Anopheles gambiae*, decarboxylates glutamic acid to produce γ-amino butyric acid but exhibits no activity toward aspartic acid. In addition, that research group identified Q377 to be located in the active site of *Ae. aegypti* ADC, which is a highly conserved amino acid residue among other insect ADCs and appears to be critical for selectivity of aspartic acid as the substrate (Liu et al. 2012) (Table 6.2).

In *D. melanogaster*, the *black* gene encoding ADC (DGAD2) is responsible for black/dark body color phenotype (Phillips et al. 1993, 2005). The *black* mutant had a deficiency of β-alanine (Hodgetts and Choi 1974) probably due to a significant decrease in ADC activity (Phillips et al. 2005). Similarly, black body color mutant

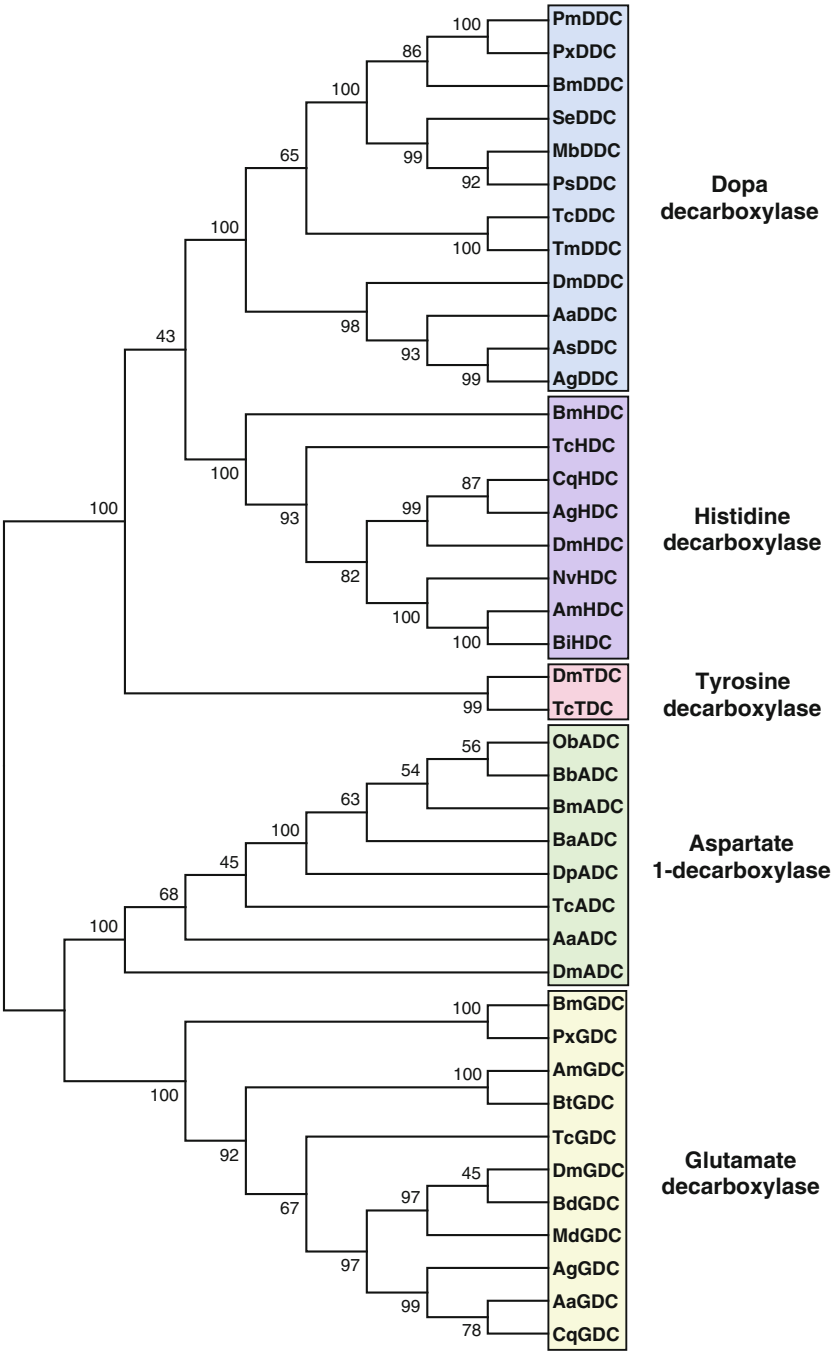

Fig. 6.3 Phylogenetic analysis of amino acid decarboxylases in insects. The amino acid sequences of DOPA decarboxylases (DDC), histidine decarboxylases (HDC), tyrosine decarboxylases (TDC), aspartate 1-decarboxylases (ADC) and glutamate decarboxylases (GDC) were obtained from GenBank. The phylogenetic tree was constructed with MEGA 6.06 software using the Neighbor-Joining method (Tamura et al. 2013). Numbers by each branch indicate results of bootstrap analysis of 5000 replications. See Table 6.2 for the accession numbers of protein sequences used in this analysis

Table 6.2 Accession numbers of insect decarboxylases used for phylogenetic analysis

Decarboxylase	Abbreviation	Species	Accession number
Dopa decarboxylase (DDC)	PmDDC	*Papilio machaon*	BAJ07588
	PxDDC	*Papilio xuthus*	NP_001299156
	BmDDC	*Bombyx mori*	NP_001037174
	SeDDC	*Spdoptera exigua*	AFG25780
	MbDDC	*Mamestra brassicae*	BAB68545
	PsDDC	*Pseudaletia separata*	BAB68549
	TcDDC	*Tribolium castaneum*	ABU25222
	TmDDC	*Tenebrio molitor*	BAA95568
	DmDDC	*Drosophila melanogaster*	NP_724164
	AaDDC	*Aedes aegypti*	AAC31639
	AsDDC	*Anopheles sinensis*	KFB39134
	AgDDC	*Anopheles gambiae*	XP_319841
Histidine decarboxylase (HDC)	BmHDC	*Bombyx mori*	XP_012551888
	TcHDC	*Tribolium castaneum*	XP_975682
	CqHDC	*Culex quinquefasciatus*	EDS34715
	AgHDC	*Anopheles gambiae*	EAA14857
	DmHDC	*Drosophila melanogaster*	CAA49989
	NvHDC	*Nasonia vitripennis*	XP_008202389
	AmHDC	*Apis melifera*	XP_392129
	BiHDC	*Bombus impatiens*	XP_012245143
Tyrosine decarboxylase (TDC)	DmTDC	*Drosophila melanogaster*	AAM70810
	TcTDC	*Tribolium castaneum*	EFA10808
Aspartate 1-decarboxylase (ADC)	ObADC	*Operophtera brumata*	KOB74239
	BbADC	*Biston betularia*	AEP43793
	BaADC	*Bicyclus anynana*	AEQ77286
	DpADC	*Danaus plexippus*	EHJ66406
	TcADC	*Tribolium castaneum*	ABU25221
	AaADC	*Aedes aegypti*	EAT40747
	DmADC	*Drosophila melanogaster*	NP_476788
	BmADC	*Bombyx mori*	AJQ30182
Glutamate decarboxylase (GDC)	BmGDC	*Bombyx mori*	XP_004925034
	PxGDC	*Papilio xuthus*	KPI96691
	AmGDC	*Apis melifera*	XP_391979
	BtGDC	*Bombus terrestris*	XP_012170748
	TcGDC	*Tribolium castaneum*	EFA06442
	DmGDC	*Drosophila melanogaster*	CAA53791
	BdGDC	*Bactrocera dorsalis*	XP_011201397
	MdGDC	*Musca domestica*	XP_011296520
	AgGDC	*Anopheles gambiae*	EAA11955
	AaGDC	*Aedes aegypti*	EAT35903
	CqGDC	*Culex quinquefasciatus*	EDS27073

strains of *T. castaneum* exhibit decreased concentrations of both β-alanine and NBAD as well as a higher level of dopamine when compared with those of wild-type beetles (Kramer et al. 1984). RNAi for *TcADC* resulted in a dark pigmented body color (Fig. 6.2d) and a significantly lower NBAD content compared to control animals like that seen in the *T. castaneum black* mutant strains (Arakane et al. 2009). Injection of β-alanine, the expected product of the reaction catalyzed by ADC, into the *black* mutant strains and ds*TcADC*-treated animals rescued the black body color phenotypes and restored the normal reddish-brown cuticle coloration (Kramer et al. 1984; Arakane et al. 2009). In addition, the *black* mutation affected the puncture resistance of the cuticle by delaying sclerotization (Roseland et al. 1987). Furthermore, dynamic mechanical analysis indicated less cross-linked cuticles from the black body color mutant and ds*TcADC*-treated animals (Arakane et al. 2009). These results suggested that the *black* mutants of *T. castaneum*, like the *D. melanogaster black* mutant, have a mutation(s) that causes loss of function of ADC, resulting in a depletion of β-alanine used for NBAD synthesis. Solid-state ^{13}C-nuclear magnetic resonance difference spectroscopy was used to determine the presence of melanin in the *black* mutant of *T. castaneum* (Kramer et al. 1989, 1995). It was estimated that 1–2 % of the organic components in the cuticle of the *black* mutant are attributable to eumelanin. The high levels of dopamine relative to the corresponding levels in the wild-type strain led to an increased production of eumelanin when the excess dopamine was oxidized in the *black* mutant (Kramer et al. 1984; Roseland et al. 1987).

Recently, Dai et al. (2015) reported that a mutation of the *ADC* gene (*BmADC*) is responsible for the black pupal (*bp*) mutant phenotype of *B. mori*, which exhibits melanization specifically in the pupal stage. In the *bp* mutant, like that seen in the *black* mutants of *T. castaneum*, there were depleted levels of *BmADC* transcripts, β-alanine and NBAD as well as accumulation of dopamine. Injection of β-alanine into the *bp* mutant reverted the dark color pattern to the wild-type pattern. RNAi for *BmADC* in the wild-type strain, furthermore, led to a melanic pupal phenotype similar to the *bp* mutant. All of these results indicated that ADC plays a role in cuticle pigmentation and sclerotization. Loss of function of ADC results in a depletion of β-alanine used for NBAD synthesis resulting in the accumulation of abnormally high levels of dopamine, which are then used for dopamine melanin production during tanning at the expense of NBAD quinone-mediated cuticle protein cross-linking and pigmentation.

6.2.4 Arylalkylamine N-Acetyltransferase (AANAT)

Arylalkylamine *N*-acetyltransferases (AANATs) belong to a large Gcn5-related acetyltransferase (GNAT) superfamily, which catalyze the transacylation between acetyl-CoA and arylalkylamines (Dyda et al. 2000; Vetting et al. 2005). AANAT has been extensively studied as a key enzyme for pineal hormone melatonin synthesis, which regulates circadian rhythms in mammals (Arendt et al. 1995; Evans 1989;

Klein 2007). AANAT activity is rate-limiting and it acetylates serotonin to form *N*-acetylserotonin in the vertebrate pineal organ, which is then methylated by hydroxyindole-*o*-methyltransferase to melatonin.

In contrast to mammals, the availability of whole genome sequences from several insect species has revealed that there is a large number of genes encoding AANAT or AANAT-like proteins in their genomes (Mehere et al. 2011; Han et al. 2012; Barbera et al. 2013; Hiragaki et al. 2015), suggesting a functional diversity of AANATs in insects. For example, melatonin has also been detected in insects such as *D. melanogaster*, *B. mori*, *A. pisum*, the migratory locust, *Locusta migratoria*, and the American cockroach, *Periplaneta americana* (Vivien-Roels et al. 1984; Finocchiaro et al. 1988; Hintermann et al. 1995; Itoh et al. 1995a, b; Hardie and Gao 1997). AANAT activity in melatonin synthesis was correlated with a circadian rhythm and seasonal photoperiodism (Itoh et al. 1995b; Bembenek et al. 2005; Vieira et al. 2005; Barbera et al. 2013). In mammals, several neurotransmitter arylalkylamines (e.g. octopamine, dopamine and serotonin) are inactivated by monoamine oxidase (MAO), whereas AANATs appear to metabolize arylalkylamines in insects because there is little or no MAO activity in their nervous tissues (Smith 1990; Amherd et al. 2000; Sloley 2004).

In insect cuticle tanning, AANAT apparently *N*-acetylates dopamine to form NADA, which is one of major precursors for quinone-mediated pigmentation and sclerotization (Fig. 6.2; Andersen 1974, 2005, 2010; Hopkins and Kramer 1992). Although the properties of insect AANATs including pH-activity profile, substrate specificity, kinetic parameters and site-direct mutagenesis to identify residues that participate in its catalysis have been studied (Hintermann et al. 1996; Brodbeck et al. 1998; Mehere et al. 2011; Han et al. 2012; Dempsey et al. 2014), information about the functional importance of AANATs in cuticle tanning is rather limited. In *B. mori*, the *melanism* (*mln*) mutant, in which the *AANAT* (*Bm-iAANAT*) gene is disrupted, shows a darker body pigmentation in the head, thoracic legs, spiracle, claw hook of prolegs and anal plate of larvae as well as in the entire body of adults (Dai et al. 2010; Zhan et al. 2010). Confirmation that a mutation of the *Bm-iAANAT* was responsible for a dark color integument in the *mln* mutant was obtained by an RNAi experiment. Injection of dsRNA for *Bm-iAANAT* into wild-type pupae resulted in the adults exhibiting a darker body pigmentation similar to that of the *mln* mutant (Zhan et al. 2010). In contrast, ectopic expression of *Bm-iAANAT* altered the dark/black coloration of neonatal larvae and adult antennae as well as larval tracheae of *B. mori* to light brown (Osanai-Futahashi et al. 2012a). This coloration change was also evident in the *B. mori* black striped strain *striped*, which has a wide black stripe in each segment. The black stripes became light grey in *Bm-iAANAT*-overexpressing lines. All of these results support the hypothesis that *N*-acetylation of dopamine decreases the availability of dopamine for dopamine-melanin production.

The dopamine content in the dark pigmented tissues (e.g. head, thoracic legs and anal plate) from the *mln* mutant of *B. mori* was two times higher than that from the wild-type strain (Dai et al. 2010). More recently, the same research group reported additional information about the catecholamine content in the *mln* mutant (Qiao et al. 2012). In the head of the larvae, dopamine and NBAD levels were higher than

those of the wild-type. In the whole body of adults, dopamine content was approximately six times greater than in the wild-type, while NBAD content was nearly the same in the two strains. This may be due to significantly lower levels of *ADC* and *NBAD synthase* (*ebony*) present in the adult *mln* mutant. Little or no NADA was evident in both larval and adult tissues. These results suggest that a loss of function of *Bm-iAANAT* results in excess dopamine (and NBAD in larvae), which likely undergoes dopamine-melanin synthesis (and NBAD-pigment synthesis in larvae) in the *B. mori mln* mutant. Dynamic mechanical analysis revealed that elytra with abnormally high dopamine collected from the TcADC-deficient adult *T. castaneum* (ds*TcADC* knockdown and the *black* mutants) exhibited a higher elastic modulus, suggesting a less effectively cross-linked cuticle than that of control insects (Arakane et al. 2009; Lomakin et al. 2010, 2011). In contrast, the wings of the *mln* mutant showed a higher modulus, indicating that the *mln* wings are stiffer than those of the wild-type strain, probably because the CPs are abnormally cross-linked.

Similarly, in adult *T. castaneum*, depletion of TcAANAT1 function by RNAi resulted in dark pigmentation of the entire body including pronotum, ventral abdomen, elytron and veins of the hindwing (Tomoyasu et al. 2009). In addition, dark pigments surrounding the bristles located on the inter-veins of the elytron were evident (Fig. 6.2f). The elytron is a highly modified and sclerotized forewing of beetles. There are a large number of pillar-like support structures called "trabeculae", which are located between the dorsal and ventral cuticles and contribute to the mechanical strength of the elytron (Ni et al. 2001; Chen and Wu 2013; Chen et al. 2015b; He et al. 2015; van de Kamp et al. 2015). The dark pigments in the elytra of ds*TcAANAT*-treated adults appear to be due to those pigmented trabeculae (unpublished observation), suggesting that NADA-mediated pigmentation and/or sclerotization is required for development of the trabeculae.

6.2.5 NBAD Synthase (Ebony)

The gene for Ebony encodes an enzyme catalyzing the synthesis of NBAD by conjugation of dopamine and β-alanine. The *ebony* mutant of *D. melanogaster* had already been described for its characteristic dark cuticle as early as 1923 (Bridges and Morgan 1923). Mutants in the *ebony* locus also show phenotypes of altered locomotion rhythm, vision or courtship behavior (Hotta and Benzer 1969; Kyriacou et al. 1978; Newby and Jackson 1991; Suh and Jackson 2007). Ebony (NBAD synthase) is closely related to non-ribosomal peptide synthases (NRPSs). Study of recombinant Ebony proteins of *D. melanogaster* that were expressed in an *E. coli* system revealed its biochemical properties (Richardt et al. 2003). *D. melanogaster* Ebony consists of 879 amino acids, which is divided into three domains, an activation/adenylation domain (572 aa), thiolation domain (78 aa) and amine-selecting domain (229 aa) (Hovemann et al. 1998; Richardt et al. 2003; Hartwig et al. 2014). The selectivity of amines is not very strict, and various biogenic amines, including dopamine, octopamine, histamine and serotonin, are β-alanylated (Richardt et al. 2003).

NBAD synthase can be defined with a more general name, β-alanylbiogenic amine synthase. Ebony is a novel type of NRPS-related protein that can be distinguished by its ability to rapidly conjugate the activated β-alanine and biogenic amines. The C-terminal amine selecting domain in Ebony is not homologous to any other protein with known domains, indicating a specific structural selection of the biogenic amine substrates (Hartwig et al. 2014). *ebony* is expressed in epithelial cells during cuticle sclerotization. In the visual system of *D. melanogaster*, *ebony* expression is localized in neural tissues, exclusively the neuropile and epithelial glial cells (Richardt et al. 2002, 2003). Its expression can be detected also in many regions of the brain and ventral nervous system from both larval and adult stages (Suh and Jackson 2007). In photoreception, it is thought that Ebony is regulating the neurotransmitter through the inactivation of histamine by *N*-β-alanylation (Richardt et al. 2002, 2003).

The involvement of Ebony in cuticle hardening is supported by a tradeoff relationship between the amount of NBAD and cuticle hardness, as showed in an analysis of the German cockroach, *Blattella germanica* (Czapla et al. 1990). This relationship is also demonstrated in the *black* mutant or *ADC* knockdown of *T. castaneum* such that the decreased amount of NBAD correlates with lowered levels of cross-links in the elytral cuticle (see Sect. 6.2.3; Arakane et al. 2009). A biochemical characterization of the *ebony* mutant was performed by demonstrating the incorporation of isotopically labeled β-alanine or dopamine into the pupal case or by examining the use of sources for NBAD synthesis, including β-alanine, dopamine and uracil, during cuticle formation (Jacobs 1968; Hodgetts 1972; Hodgetts and Konopka 1973; Hodgetts and Choi 1974). In both the *ebony* mutant of *D. melanogaster* and a melanotic body color mutant of the medfly, *Ceratitis capitata*, it was postulated that a defective NBAD synthase is responsible for the low level of NBAD and conversely high level of dopamine leading to a darker coloration (Wright 1987; Wappner et al. 1996). By using an *in vitro* cell free system of tissue homogenate from *C. capitata*, the enzyme activities of Ebony were directly characterized. Endogenous substrates like dopamine, norepinephrine and L-tyrosine were β-alanylated by tissue extracts from wild-type individuals, but the extract from the mutant exhibited only negligible levels of β-alanylation of these substrates (Perez et al. 2002). No *N*-β-alanyldopa was synthesized, which is consistent with the absence of *N*-β-alanyldopa in insect cuticle. It was reported in studies of *Sarcophaga* species that *N*-β-alanyl-tyrosine (Sarcophagine) and a derivative of *N*-β-alanyldopa, *N*-β-alanyl-5-*S*-glutathionyl-3-4-dihydroxyphenylalanine (5-*S*-GAD), have antibacterial activities (Leem et al. 1996; Meylaers et al. 2003). NBAD itself has an antibacterial activity comparable in strength to those of *N*-β-alanyldopa and 5-*S*-GAD (Schachter et al. 2007). The NBAD levels were increased in the hemolymph of the mealworm beetle, *Tenebrio molitor*, after bacterial challenges (Kim et al. 2000). Furthermore, NBAD synthase activity was detected in the integument of bacteria-injected *T. molitor* larvae (Schachter et al. 2007). In *C. capitata*, expression of *ebony* was induced in epidermal cells in response to bacterial challenges. After a bacterial challenge, an allele of the *ebony* mutant (e^{11}) with a cuticle deficiency (Lindsley and Grell 1968) showed susceptibility to *Serratia* infection via oral

administration (Flyg and Boman 1988). These observations imply a direct involvement of NBAD in defense reactions or probably in protection by a mechanically strong cuticle that is stabilized by protein cross-links involving NBAD.

Like other genes involved in pigmentation, insect body color pattern is associated with the pattern of *ebony* expression. NBAD is also important for production of papiliochromes that are yellowish-reddish-brownish pigments composed of NBAD and kynurenines in Papilionidae butterflies (Umebachi 1990). In larvae of *P. xuthus, ebony* is expressed in the epithelial cells underneath those cuticle regions that have a reddish-brown coloration (Futahashi and Fujiwara 2005). Expression of *ebony* is observed in red and yellow regions of the wings of *P. polytes* (Nishikawa et al. 2013). In Drosophilid species, high levels of *ebony* expression are linked to low levels of pigmentation in the thoracic trident or abdomen (Wittkopp et al. 2002a, 2009; Pool and Aquadro 2007; Takahashi et al. 2007). The same correlation was observed with the colors of butterfly wings (Ferguson et al. 2011b). In *B. mori sooty* mutants with a dark body color, *ebony* was identified as the responsible gene (Futahashi et al. 2008b). Recently, Ebony was utilized as a visible marker gene for genotyping of transgenic insects. Ubiquitous expression of *ebony* via the Gal4/UAS system in *B. mori* caused a light pigmentation in the larval body or adult antennae (Osanai-Futahashi et al. 2012a).

6.2.6 NBAD Hydrolase (Tan)

NBAD hydrolase (Tan) is a product of the *tan* gene and it shows high sequence similarity to fungal isopenicillin *N*-acyltransferase (IAT; True et al. 2005), which is involved in penicillin-G biosynthesis (Queener and Neuss 1982; Barredo et al. 1989). Like IAT, Tan appears to be expressed as a precursor protein that is activated by self-processing into two polypeptide subunits at a conserved Gly-Cys motif (e.g. Gly_{121}-Cys_{122} in DmTan; Wagner et al. 2007; Aust et al. 2010; Perez et al. 2011). One of the essential roles of Tan activity is the hydrolysis of β-alanylhistamine (carcinine) in the visual system (True et al. 2005; Wagner et al. 2007; Perez et al. 2011). In *D. melanogaster*, DmTH is localized in photoreceptor cells where it hydrolyzes carcinine to histamine for neurotransmission with the former metabolite provided by NBAD synthase (Ebony) localized in the surrounding glial cells (Richardt et al. 2002).

In insect epidermal cells, Tan hydrolyzes NBAD to form dopamine and β-alanine, which is a reverse reaction of NABD synthesis catalyzed by Ebony (Fig. 6.2; Wittkopp et al. 2002a; Wright 1987; True et al. 2005). A functional importance of *tan* in cuticle pigmentation has been reported for a few insect species including *D. melanogaster*, *B. mori* and *P. xuthus*. Disruption of the *tan* gene by mutation or *P*-element insertion caused pigmentation defects in thoracic and abdominal cuticles in adult *D. melanogaster* (True et al. 2005; Jeong et al. 2008), and ectopic expression of *tan* rescued the pigmentation phenotypes in the *tan* mutant (True et al. 2005). In addition, Jeong et al. (2008) reported that expression of both *tan* and *dopachrome*

conversion enzyme (*yellow*, see Sect. 6.2.8) genes correlated with a diversity of body pigmentation patterns between *D. yakuba* and *D. santomea*. In the latter, the loss of abdominal pigmentation involves little or no *tan* and *yellow* gene expression.

In *P. xuthus* and *B. mori*, expression of *tan* along with *laccase 2* (see Sect. 6.2.7) is strongly correlated with larval black markings/pigments (Futahashi et al. 2010). For example, the *tan* transcript was clearly detected in the black region of the eyespot marking of *P. xuthus* larvae, but it was absent in the reddish-brown region of this marking. In the *B. mori rouge* (*ro*) mutant, the larval black markings are light brown. In this mutant, the *tan* cDNA lacks exon 2, resulting in a premature stop codon insertion. The predicted Tan protein is missing a large portion including a conserved self-processing site, suggesting that *tan* is responsible for the larval body color phenotype in the *ro* strain (Futahashi et al. 2010). Taken together, these results suggest that Tan plays a role in cuticle pigmentation through hydrolysis of NBAD to provide dopamine, which is a major precursor for melanin production. Tan may also be critical for the cuticle's mechanical properties because its activity influences the level of NBAD, which serves as a cuticle protein cross-linking agent. Further study is required to confirm this hypothesis.

6.2.7 Laccase (Lac)

Since the first description of laccase from the Japanese lacquer tree, *Rhus vemicifera* (Yoshida 1883), this enzyme has been extensively studied, and now there is a large accumulation of knowledge about its enzymatic properties, gene function and structure (Nakamura and Go 2005; Sharma et al. 2007). Proteins like laccase, ascorbate oxidase or the bacterial proteins, CueO or CumA, have a common feature in that these proteins are composed of three repeats of cupredoxin domains. They form a sub-protein group of three domain multicopper oxidases (3dMCO). In insects, laccase is regarded as one of the key enzymes for cuticle sclerotization and pigmentation. The roles of laccase are thought to be dependent on its enzyme activity to oxidize *ortho*-diphenols to the corresponding quinones. Since the first characterization of laccase-like activity in *Drosophila virilis* (Ohnishi 1954; Yamazaki 1969), laccase-like proteins were partially purified from the integument from several insect species (Yamazaki 1972; Andersen 1978; Barrett and Andersen 1981; Barrett 1987a, b; Thomas et al. 1989). Until the end of 1980s, the enzymatic properties of these proteins were characterized in some detail (Ashida and Yamazaki 1990; Barrett 1991; Dittmer and Kanost 2010). In 2004, the first cDNA sequences for laccase-like proteins from lepidopteran and dipteran species were reported (Dittmer et al. 2004). In this study, three genes, *laccase 1* and *laccase 2* from *M. sexta* (*MsLac1* and *MsLac2*) and *laccase 1* (*AgLac1*) of *An. gambiae* were identified. One characteristic of the insect laccase-like proteins is a methionine residue at the T1 copper center (Met716 of MsLac1, Met728 of MsLac2 and Met948 of AgLac1), whereas in laccases from plants and fungi this residue position has a phenylalanine or leucine.

Other characteristics of the insect laccase-like proteins are the N-terminal extensions that include an N-terminal signal peptide and a cysteine-rich region in all three of the proteins. By using the efficient RNAi system of *T. castaneu*m, loss of function phenotypes were analyzed. dsRNA for *laccase 2* (*TcLac2*) was injected into the hemocoel of individuals at various stages. After ecdysis to larva, pupa and adult, the new cuticles of the dsRNA-injected individuals were untanned. Also in the adult, a severe wing deformation was also observed (Arakane et al. 2005).

By the early 1990s, three types of phenol-oxidizing enzymes had been found in insect cuticle. They were designated as a tyrosinase-type phenoloxidase, laccase-type phenoloxidase (or laccase) and granular phenoloxidase. There had been a long discussion on the roles and classification of these cuticular enzymes (Ashida and Yamazaki 1990; Barrett 1991). Since they all have activity to oxidize *o*-diphenols, their involvement in cuticle pigmentation and hardening during development has been one of the important areas of investigation in insect cuticle physiology. It was shown that tyrosinase-type phenoloxidase is synthesized in the hemocytes, then secreted into the hemolymph, and finally transported into the cuticle (Ashida and Brey 1995; Asano and Ashida 2001). Granular-PO (GPO) was originally purified from granules in the larval cuticle of *M. sexta* by using a preparative electrophoretic gel in the presence of sodium dodecyl sulfate (see Sect. 6.4; Hiruma and Riddiford 1988). As observed in several other insect species, cuticular melanin was developed within premelanin granules deposited into the outer procuticle (Kayser-Wegmann 1976; Curtis et al. 1984). The GPO is thought to be responsible for production of melanin in such structures, but the gene for the granular enzyme has not been identified. To show the involvement of proPOs and laccase-like MCOs in cuticle formation during development, the phenotypes resulting from knockdown of these genes by RNAi were compared in *T. castaneum* (Arakane et al. 2005). dsRNAs for *proPO* genes (both of *TcTyr1* and *TcTyr2*), *laccase 1* (*TcLac1*) and *TcLac2* were injected. In contrast to the result that knockdown of *TcLac2* induced severe defects in pigmentation and abnormal adult shapes, no visible phenotypes were observed when *TcTyr1*, *TcTyr2* and *TcLac1* were knocked down. This result strongly suggests that the gene for the tyrosinase-type protein, proPO, is not involved in the process of cuticle pigmentation and hardening during development.

Since the study of laccase 2 in *T. castaneum*, the RNAi method has been adopted for use in other species from multiple orders to study the involvement of *laccase 2* genes in cuticle formation (Niu et al. 2008; Elias-Neto et al. 2010; Futahashi et al. 2010; Ye et al. 2015). In all cases, pigmentation was suppressed. In several cases, the cuticle showed an abnormal shape and became mechanically weak (Arakane et al. 2005; Ye et al. 2015). RNAi for *TcLac2* also reduced egg hatching rates at low humidity, demonstrating that the enzyme is crucial for sclerotization of the serosal cuticle and for embryonic desiccation resistance (Jacobs et al. 2015). In the larval integument of *B. mori*, the spatial patterns of *BmLac2* expression exhibit a close correlation with those of pigmentation. The genes for substrate synthesis (*TH, DDC, tan* and *ebony* in Fig. 6.1) are also expressed strongly in the areas of black and reddish pigmentation (Futahashi and Fujiwara 2005, 2007; Futahashi et al. 2010). In the RNAi study on *TcLac2* from *T. castaneum* (Arakane et al. 2005), the authors

also described its characteristic gene structure, indicating the formation of two splice variants, A- and B-type TcLac2. The protein products from the two variants have the same N-terminal 491 amino acids, but the remaining C-terminal portion is encoded by a distinct set of exons. The C-terminal variable region includes copper-binding sites that are indispensable for oxygen binding inside the active center. It is assumed that the two isoforms have different enzymatic properties for versatility in functions. The presence of A-type and B-type isoforms is also found in *An. gambiae* (Gorman et al. 2008) and at least three variants are found in *D. melanogaster* (Flybase, http://flybase.org/; Asano et al. in preparation). Although it has not been proven experimentally, the gene structure of *BmLac2* implies that the A- and B-types can be produced by similar splicing patterns (Yatsu and Asano 2009). The expressions of A- and B-type variants were compared by PCR in *T. castaneum* and *An. gambiae*. In each case, the temporal peak of expression is slightly different between the two isoforms (Arakane et al. 2005; Gorman et al. 2008), indicating that each has a unique function related to a specific timing. The knockdown of each isoform in *T. castaneum* led to lethal phenotypes, but the deformation of the cuticle was more severe in the knockdown of the A-type isoform.

Amino acid sequence analysis was performed for a laccase-like enzyme that was purified from the newly ecdyzed pupae of *B. mori* (Yatsu and Asano 2009). The procedure for the purification was a modified version of the previous report by Yamazaki (1972). Like the previous study, trypsin was used for solubilization of laccase activity. Since laccase is attached to the cuticle matrix very tightly, proteases or denaturing regents are needed to break down the cuticle structure anchoring the laccase protein. The purity was increased from three bands in the previous study to only a single band (70 kDa) by analysis with SDS-PAGE. The N-terminal sequence of the purified enzyme was NPALS that corresponds to Asn147-Ser151 of the full-length putative polypeptide deduced from the cDNA sequence of *BmLac2* (Fig. 6.4). The mass spectrometric identification of tryptic fragments from the purified enzyme (trypsin-solubilized *B. mori* laccase 2, Bm-tLac2) failed to identify peptides corresponding to 146 amino acids from the N-terminal methionine, suggesting that the purified enzyme had lost the N-terminal portion during treatment with trypsin. A similar result was reported in the analysis Lac2 from *M. sexta*. The full-length and N-terminally truncated (**Δ**106) recombinant proteins exhibit similar catalytic parameters when NADA is used as substrate, indicating that the presence of the N-terminal portion does not have a significant influence on the enzymatic activity (Dittmer et al. 2009). After these studies, the molecular properties of the recombinant laccase 2 proteins of *T. castaneum* and *An. gambiae* were determined (Gorman et al. 2012). They exhibit the same pattern of *Km* values with dopamine and NADA being the more preferred substrates than DOPA and NBAD, respectively. The pH optima of enzyme activities are also weakly acidic and the A-type isoforms exhibit higher pH optima for DOPA and dopamine than the B-type isoforms. The four recombinant proteins show similar kinetic parameters, but a notable difference was seen in the case of TcLac2B with its k_{cat} values much larger than the values determined for TcLac2A. The variation in pH optima and substrate preference in the two isoforms may reflect the environment in which each isoform is localized.

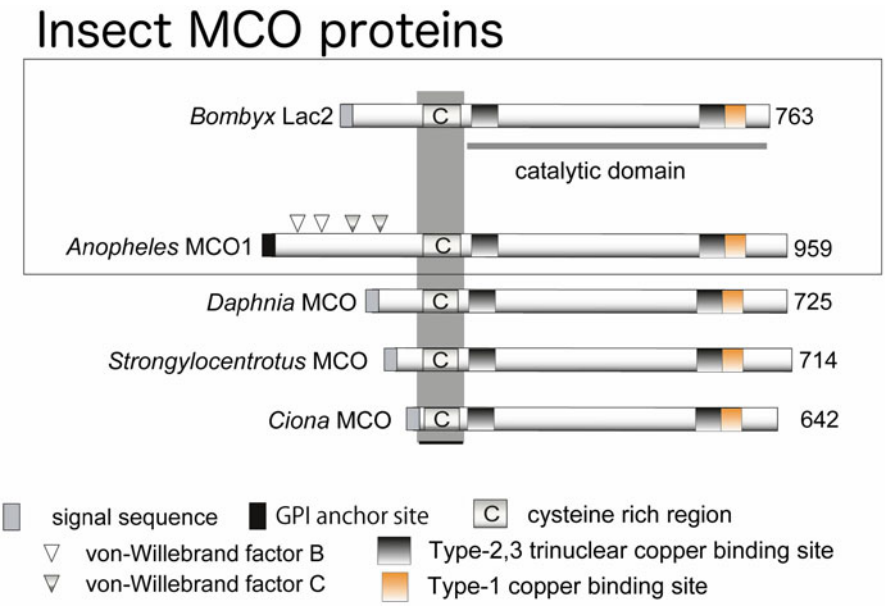

Fig. 6.4 Schematic domain structures of 3dMCOs from invertebrates. Two proteins from insects and three proteins from other invertebrate species are shown. Structural characteristics are highlighted including the signal sequence, catalytic domain, cysteine-rich region, GPI anchor site, von-Willebrand factor domains and copper-binding sites. Numbers indicate length of proteins (in numbers of amino acids)

Since the early studies, there have been observations that imply an active regulation of laccase function during the process of cuticle construction in *D. virillis* (Yamazaki 1969) and the Australian sheep blowfly, *Lucilia cuprina* (Barrett 1987b). In *B. mori*, little laccase activity was detected in the cuticle of newly ecdysed pupa, but the activity increased to a maximum at 4 h after pupal ecdysis (Yamazaki 1972). A similar result was obtained by *in situ* staining of the pupal cuticle with dopamine (Yatsu and Asano 2009). It had been suggested that laccase is synthesized as an inactive precursor and accumulates in the pupal cuticle before ecdysis. After pupal ecdysis, the precursor may be activated through processing or interactions with other molecule(s). A precursor-like protein was extracted from the cuticle of newly ecdyzed pupae of *B. mori* by treatment with α-chymotrypsin. When examined by activity staining after native PAGE, only a faint activity band was detected, but after *in vitro* treatment with trypsin, an increased activity was detected (Ashida and Yamazaki 1990; Asano et al. 2014). The purified α-chymotrypsin-solubilized BmLac2 (Bm-cLac2) protein was shown to be an active enzyme, but the specific activity was increased 17-fold after treatment with trypsin. During that treatment, peptide bonds in the C-terminal region (Lys677-Gln678 and Arg674-His675) were cleaved. Although it is not an inactive precursor, Bm-cLac2 appears to preserve the character of the hypothetical inactive-precursor in that it has the potential to become

substantially more active. For the precise control of a successful completion of the entire ecdysial process, it is reasonable to utilize multiple systems to regulate laccase 2 activity. It is hypothesized that laccase 2 can become a much more active enzyme by some activating factors through proteolytic processing or other modes of interactions (Asano et al. 2014).

6.2.8 Dopachrome Conversion Enzyme (Yellow)

The *yellow* gene, which was named for a yellowish body color in a loss of function phenotype of *D. melanogaster* (Nash and Yarkin 1974), appears to be one of the most rapidly evolving gene families that generates functionally diverse paralogs. This family includes the major royal jelly proteins (MRJPs) of the honeybee, *Apis mellifera*, which are the most abundant proteins found in royal jelly (Schmitzova et al. 1998; Albert and Klaudiny 2004). The *yellow* genes encoding MRJPs and MRJP-like (MRJPL) proteins have been identified not only in honeybees but also in several other hymenopteran species. Those *MRJP* and *MRJPL* genes are generally arranged in a cluster (except for a single copy of *MRJPL* in some species) of closely linked genes, which is located between *yellow-e3* and *yellow-h* (most common gene order is *yellow-g*, *−g2*, *−e*, *−e3*, *MRJPs* or *MRJPLs*, *−h*) in their genomes (Ferguson et al. 2011a; Kupke et al. 2012; Buttstedt et al. 2014). Although the *MRJPs* or *MRJPLs* locus is highly conserved, recent studies including phylogenetic analysis suggest that the gene clades in *A. mellifera*, the jewel wasp, *Nasonia vitripennis*, and the Argentine ant, *Linepithema humile*, have independently evolved in their lineage (Fig. 6.5; Werren et al. 2010; Smith et al. 2011; Buttstedt et al. 2014). MRJP proteins were detected in a variety of tissues including brain, venom glands and larval hemolymph and in different developmental stages in *A. mellifera* (Kucharski et al. 1998; Schmitzova et al. 1998; Drapeau et al. 2006; Peiren et al. 2008; Randolt et al. 2008; Hojo et al. 2010), suggesting that MRJPs have other physiological functions in addition to a nutritional role due to their high content of essential amino acids (Table 6.3).

Yellow and *MRJP/MRJPL* comprise a gene family in insects. This gene family of the melanin pathway appears to be insect specific and deviates from the pathway common to other animals. The number of genes in insects, whose genomes are well characterized, is in the range of 10–26 in species such as *D. melanogaster* (Maleszka and Kucharski 2000; Drapeau 2001), *T. castaneum* (Arakane et al. 2010), *B. mori* (Xia et al. 2006), *P. xuthus* (Futahashi et al. 2012), *A. mellifera* (Drapeau et al. 2006) and *N. vitripennis* (Werren et al. 2010). The encoded Yellow and MRJP/MRJPL proteins have been divided into at least ten subgroups based on sequence similarity and phylogenetic analysis (Fig. 6.5; Ferguson et al. 2011a). Although a large number of *yellow* and *MRJP/MRJPL* genes has been identified in different insect species, the physiological function(s) of most of these genes has not yet been determined.

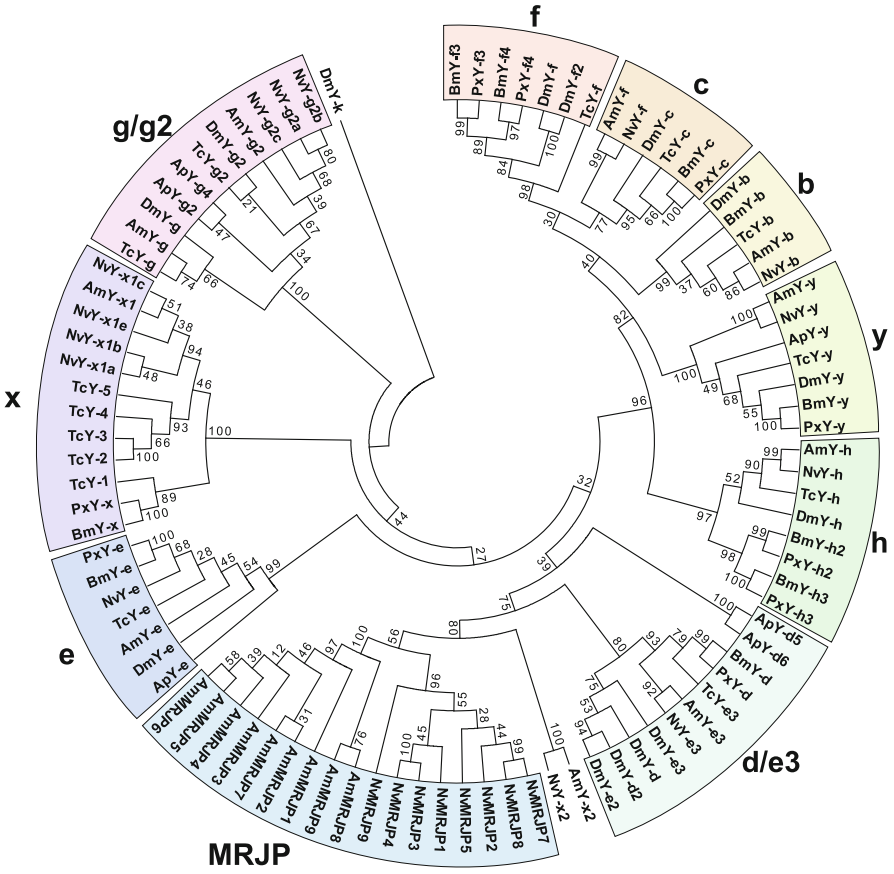

Fig. 6.5 Phylogenetic analysis of Yellow and Yellow-like proteins in insects. ClustalW software was used to perform multiple sequence alignments prior to phylogenetic tree analysis. The phylogenetic tree was constructed with MEGA 6.06 software using the Neighbor-Joining method (Tamura et al. 2013). Numbers by each branch indicate results of bootstrap analysis of 5000 replications. See Table 6.3 for the accession numbers of protein sequences used in this analysis

In the cuticle tanning pathway, a *yellow* gene encodes dopachrome conversion enzyme (DCE) that converts dopachrome and dopaminechrome to dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), respectively, during melanin biosynthesis (Fig. 6.2). DCE accelerates melanin synthesis in insects such as *D. melanogaster, B. mori, P. xuthus* and *T. castaneum* (Wittkopp et al. 2002b; Futahashi and Fujiwara 2007; Futahashi et al. 2008a; Arakane et al. 2010; Ito et al. 2010). The Yellow protein is critical for cuticle pigmentation in several species. In *D. melanogaster*, for instance, *yellow* (*DmY-y*) is required for black melanin production, because mutation of *DmY-y* leads to the light brown/yellowish cuticle and localization of DmY-y protein is correlated with black pigmentation patterns in adult cuticle (Wittkopp et al. 2002a, b, 2003a, 2009; Gompel et al. 2005). Riedel

Table 6.3 Accession numbers of insect Yellow and Yellow-like proteins used for phylogenetic analysis

Species	Abbreviation	Protein	Accession number
Tribolium castaneum	TcY-b	Yellow-b	ACY71055
	TcY-c	Yellow-c	ACY71056
	TcY-e	Yellow-e	ACY71058
	TcY-e3	Yellow-e3	ACY71057
	TcY-f	Yellow-f	ACY71059
	TcY-g	Yellow-g	ACY71060
	TcY-g2	Yellow-g2	ACY71061
	TcY-h	Yellow-h	ACY71062
	TcY-y	Yellow-y	ACY71063
	TcY-1	Yellow-1	ACY71064
	TcY-2	Yellow-2	ACY71065
	TcY-3	Yellow-3	ACY71066
	TcY-4	Yellow-4	ACY71067
	TcY-5	Yellow-5	ACY71068
Drosophila melanogaster	DmY-b	Yellow-b	NP_523586
	DmY-c	Yellow-c	NP_523570
	DmY-d	Yellow-d	NP_523820
	DmY-d2	Yellow-d2	NP_611788
	DmY-e2	Yellow-e2	NP_650289
	DmY-e3	Yellow-e3	NP_650288
	DmY-e	Yellow-e	NP_524344
	DmY-f	Yellow-f	NP_524335
	DmY-f2	Yellow-f2	NP_650247
	DmY-g	Yellow-g	NP_523888
	DmY-g2	Yellow-g2	NP_647710
	DmY-h	Yellow-h	NP_651912
	DmY-y	Yellow-y	NP_476792
	DmY-k	Yellow-k	NP_648772
Bombyx mori	BmY-b	Yellow-b	BGIBMGA014224-TA
	BmY-c	Yellow-c	ABC96696
	BmY-d	Yellow-d	ABC96694
	BmY-e	Yellow-e	BAI39592
	BmY-f3	Yellow-f3	NP_001037424
	BmY-f4	Yellow-f4	NP_001037428
	BmY-h2	Yellow-h2	BGIBMGA007255-TA
	BmY-h3	Yellow-h3	BGIBMGA007256-TA
	BmY-y	Yellow-y	BAH11146
	BmY-x	Yellow-x	NP_001037430

(continued)

Table 6.3 (continued)

Species	Abbreviation	Protein	Accession number
Papilio xuthus	PxY-c	Yellow-c	BAM18241
	PxY-d	Yellow-d	BAM18620
	PxY-e	Yellow-e	BAM17806
	PxY-f3	Yellow-f3	BAM18870
	PxY-f4	Yellow-f4	BAM18225
	PxY-h2	Yellow-h2	BAM17969
	PxY-h3	Yellow-h3	BAM17970
	PxY-y	Yellow-y	BAF73474
	PxY-x	Yellow-x	BAM18176
Apis melifera	AmY-b	Yellow-b	XP_006569902
	AmY-e3	Yellow-e3	ABB82366
	AmY-e	Yellow-e	XP_003249426
	AmY-f	Yellow-f	NP_001011635
	AmY-g	Yellow-g	XP_396824
	AmY-g2	Yellow-g2	XP_006559006
	AmY-h	Yellow-h	ABB81847
	AmY-y	Yellow-y	NP_001091693
	AmY-x1	Yellow-x1	XP_006565008
	AmY-x2	Yellow-x2	XP_001122824
	AmMRJP1	Major Royal Jelly Protein (MRJP) 1	NP_001011579
	AmMRJP2	MRJP2	NP_001011580
	AmMRJP3	MRJP3	NP_001011601
	AmMRJP4	MRJP4	NP_001011610
	AmMRJP5	MRJP5	NP_001011599
	AmMRJP6	MRJP6	NP_001011622
	AmMRJP7	MRJP7	NP_001014429
	AmMRJP8	MRJP8	NP_001011564
	AmMRJP9	MRJP9	NP_001019868

(continued)

Table 6.3 (continued)

Species	Abbreviation	Protein	Accession number
Nasonia vitripennis	NvY-b	Yellow-b	NP_001154989
	NvY-e3	Yellow-e3	NP_001154982
	NvY-e	Yellow-e	NP_001154985
	NvY-f	Yellow-f	NP_001154968
	NvY-g2a	Yellow-g2a	XP_001603544
	NvY-g2b	Yellow-g2b	NP_001154986
	NvY-g2c	Yellow-g2c	XP_001603573
	NvY-h	Yellow-h	NP_001153394
	NvY-y	Yellow-y	NP_001154977
	NvY-x1a	Yellow-x1a	XP_001601771
	NvY-x1b	Yellow-x1b	NP_001155026
	NvY-x1c	Yellow-x1c	NP_001154990
	NvY-x1e	Yellow-x1e	XP_001607252
	NvY-x2	Yellow-x2	XP_001601022
	NvMRJP1	MRJP1	XP_001603404
	NvMRJP2	MRJP2	NP_001155025
	NvMRJP3	MRJP3	NP_001154981
	NvMRJP4	MRJP4	NP_001154980
	NvMRJP5	MRJP5	NP_001154979
	NvMRJP7	MRJP7	NP_001154975
	NvMRJP8	MRJP8	NP_001154974
	NvMRJP9	MRJP9	NP_001154978
Acyrthosiphon pisum	ApY-d5	Yellow-d5	XP_001942700
	ApY-d6	Yellow-d6	XP_001942648
	ApY-e	Yellow-e	XP_001948479
	ApY-g2	Yellow-g2	XP_001945004
	ApY-g4	Yellow-g4	XP_001944949
	ApY-y	Yellow-y	NP_001165848

et al. (2011) demonstrated that overexpression of *DmY-y* altered coloration of the adult wing cuticle from brown to black. In addition, the multi-ligand endocytic receptor Megalin (Mgl) plays a role in promoting internalization/endocytosis of DmY-y. Loss of function of *Mgl* caused excess DmY-y, resulting in a black pigmented wing similar to that seen in the adult in which *DmY-y* is overexpressed. This result suggests that, in addition to the spatial expression, DmY-y function is regulated by a cellular process such as an endocytosis for cuticle coloration of *D. melanogaster*. Further studies are needed to know whether this is the case in other insect species.

Like *DmY-y*, Yellow is also important for body pigmentation in lepidopteran species. Expression of *yellow* in the swallowtail species *P. xuthus* (*PxY-y*), *P. machaon* (*PmY-y*) and *P. polytes* (*PpY-y*) is correlated with black body markings of their larval cuticles (Futahashi and Fujiwara 2007; Futahashi et al. 2010; Shirataki et al. 2010). Futahashi et al. (2012) utilized microarray analysis to screen for marking-

specific genes using six markings at 11 different stages of *P. xuthus* larvae and all of the *yellow* gene family members were identified except for *yellow-b*. Interestingly, *yellow-h3* (*PxY-h3*) exhibited an expression pattern of development very similar to that of *PxY-y*. In addition, *PxY-h3* was expressed in the black pigmented markings, indicating that, like *PxY-y*, it also is involved in marking-specific melanin synthesis in *P. xuthus*. In *B. mori*, *yellow-y* (*BmY-y*) and *yellow-e* (*BmY-e*) had been characterized as genes responsible for the body color mutants, *ch* (*chocolate*) and *bts* (brown head and tail spot), respectively (Futahashi et al. 2008b; Ito et al. 2010). The larval body color of the *ch* mutant strain is a reddish brown instead of a normal black color, whereas the *bts* mutant strain exhibited a reddish brown larval head and anal plates instead of the white coloration found in the wild-type strain. These results suggested that both BmY-y and BmY-e play critical roles in larval pigmentation of *B. mori*.

In *T. castaneum*, 14 *yellow* genes have been identified and their different expression patterns indicated that yellow genes have diverse functions (Arakane et al. 2010). Functional importance of *yellow* in cuticle pigmentation has been identified in several insect species as described above. Interestingly, knockdown of the *yellow* (*TcY-y*) by RNAi had no effect on body pigmentation in the larva, pupa and adult except for black pigmentation in the pterostigma of the hindwing (Fig. 6.2g; Arakane et al. 2010), suggesting that TcY-y is not critical for *T. castaneum* body wall cuticle pigmentation. Yellow-f (DmY-f) and -f2 (DmY-f2) in *D. melanogaster* showed a DCE activity required for melanin synthesis (Han et al. 2002). However, unlike *PxY-y* and *PxY-h3*, *yellow-f3* (*PxY-f3*) and *-f4* (*PxY-f4*), which are homologs of *DmY-f/-f2* in *P. xuthus*, were not up-regulated in the larval black markings (Futahashi et al. 2012). RNAi for *T. castaneum* homolog of *DmY-f/-f2* (*TcY-f*) had no effect on body pigmentation of the larva, pupa and pharate adult. The resulting pharate adults, however, were unable to shed their exuviae and died entrapped in their old pupal cuticle (Arakane et al. 2010). These results suggested that Yellow-f-related genes might have other functions in addition to melanin production in *P. xuthus* and *T. castaneum*.

Recently, a novel anti-dehydration function of *yellow-e* (*TcY-e*) in *T. castaneum* has been reported (Noh et al. 2015a). RNAi for *TcY-e* had no effect on larval, pupal and pharate adult cuticle pigmentation. However, the resulting adults died shortly after eclosion due to dehydration, and the lethality was prevented by high humidity. The body color of the high humidity-rescued adults, like that observed in the *B. mori bts* mutant, was significantly darker than that of control adults (Fig. 6.2f), suggesting that TcY-e plays a role not only in body pigmentation but also has a vital waterproofing function in *T. castaneum* adults. In contrast to loss of function of *yellow*, which causes a lighter body color in many insect species, depletion of *yellow-e* function results in a darker body pigmentation in both *B. mori* and *T. castaneum*. The most prominent function of the *yellow* gene is in the production of black pigment in a variety of insects. However, the function of the other members of the family is largely obscure. Further study is required to understand the function of the *yellow* paralogs across different insect species including the molecular mechanisms for the cause of the dark body pigmentation in *yellow-e* deficient insects.

6.2.9 Structural Cuticle Proteins (CPs)

In arthropods, structural cuticular proteins (CPs) play important roles in determining the diverse physical properties of the cuticle depending on developmental stages as well as different body regions as a result of interactions with other CPs and the structural biopolymer chitin (Neville 1993). Recent studies and the availability of fully sequenced and annotated genomes of several insect species such as *A. mellifera* (Honeybee Genome Sequencing Consortium. 2006), *D. melanogaster* (Karouzou et al. 2007), *T. castaneum* (Dittmer et al. 2012; *Tribolium* Genome Sequencing Consortium. 2008), *An. gambiae* (Cornman et al. 2008), *B. mori* (Futahashi et al. 2008a) and *N. vitripennis* (Werren et al. 2010) indicate that there are large numbers of genes encoding CP-like proteins in their genomes (see Chap. 1 in this book for details about CPs). Indeed, more than 200 putative *CP* genes have been identified in *D. melanogaster* and *An. gambiae* (Ioannidou et al. 2014), and these genes comprise ~2 % of the predicted protein-coding genes in the latter species.

CPs have been classified into thirteen distinct families as defined by unique amino acid sequence motifs characteristic of each of these families (Willis 2010; Willis et al. 2012; Ioannidou et al. 2014). The largest cuticular protein family is the CPR family whose members contain a conserved amino acid sequence known as the Rebers & Riddiford (R&R) consensus motif (Rebers and Riddiford 1988). The R&R motif contains a chitin-binding domain (chitin-bind-4 domain, PF00379 in the Pfam database) that apparently helps to coordinate the interactions between chitin fibers and the proteinaceous matrix (Rebers and Willis 2001; Togawa et al. 2004, 2007; Qin et al. 2009). CPR proteins containing the RR-1 motif have been found primarily in soft and flexible cuticle, whereas the proteins containing the RR-2 motif have been found mostly in hard and rigid cuticle. The third class of CPR proteins, RR-3, has been identified in only a few insect species and is a very minor group whose distinguishable features have not yet been well defined (Andersen 2000).

During cuticle tanning, some of CPs are cross-linked by quinones and/or quinone methides produced by laccase 2-mediated oxidation of *N*-acylcatechols (Hopkins and Kramer 1992; Arakane et al. 2005; Andersen 2008; Mun et al. 2015). This vital process together with dehydration occurs during each stage of development and the expression of specific CPs appears to be required for formation of diverse cuticles in different regions of the insect's body and at different developmental stages so that an appropriate combination of physical and morphological features can provide structural support, mechanical protection and mobility. However, little is known about the functional importance of individual insect CPs in the morphogenesis and mechanical properties of the cuticle.

Four major CPs, TcCPR27, TcCPR18, TcCPR4 and TcCP30, were identified in protein extracts of elytra from *T. castaneum* adults (Arakane et al. 2012; Dittmer et al. 2012; Mun et al. 2015; Noh et al. 2015b). All of these CPs are abundant in rigid cuticles including the dorsal elytron, pronotum, ventral abdomen and leg,

while they are absent or very minor in soft and flexible cuticles such as the ventral elytron, hindwing and dorsal abdomen of the adult. TcCPR27 and TcCPR18 are members of the RR-2 group of the CPR family, and are localized in both the chitinous horizontal laminae and vertical pore canals in the procuticle of rigid adult cuticle (Noh et al. 2014). RNAi for *TcCPR27* or *TcCPR18* genes caused a disorganized laminar architecture and amorphous pore canal fibers (PCFs), which led to short, wrinkled and weakened elytra (Arakane et al. 2012; Noh et al. 2014). TcCPR4, which is more highly extractable from the elytra of TcCPR27-deficient adults than are other CPs, contains an RR-1 motif (Noh et al. 2015b). TEM immunogold labeling revealed that the TcCPR4 protein is predominantly localized in the pore canals and in the vicinity of the apical plasma membrane protrusions of the procuticle. However, depletion of TcCPR27 caused mislocalization of TcCPR4. In the TcCPR27-deficient elytra, the TcCPR4 protein was distributed over the entire procuticle including the horizontal laminae, suggesting that the presence of TcCPR27 protein is critical for the specific localization of TcCPR4 protein. Loss of function of *TcCPR4* produced by RNAi caused an abnormal shape of the pore canals with amorphous PCFs in their lumen, indicating that TcCPR4 is important for determining the morphology and ultrastructure of PCFs and pore canals in rigid cuticle.

Unlike TcCPR27, TcCPR18 and TcCPR4, the mature TcCP30 protein has a low complexity amino acid sequence with a rather unique amino acid composition (36 % Glu, 21 %, His, 19 %, Arg and 16 % Gly), and lacks an R&R consensus motif (Mun et al. 2015). The function of TcCP30 is critical for adult eclosion of *T. castaneum* probably because it undergoes cross-linking during cuticle maturation. Western blotting analysis of protein extracts from the elytra and pronotum revealed that TcCP30 becomes cross-linked to TcCPR27 and TcCPR18, but not to TcCPR4, by the action of laccase 2 (Mun et al. 2015). Because TcCP30, TcCPR27 and TcCPR18 have a high histidine content (10–21 %), whereas TcCPR4 has a relatively low content (3 %), this result appears to be consistent with the hypothesis that histidine residues of CPs most likely participate in quinone- and/or quinone methide-mediated CP cross-linking (Kramer et al. 2001). All of these results indicate that the unique localization and cross-linking of specific CPs are important for morphology and ultrastructure of the exoskeleton (Fig. 6.6). Genes encoding CPs comprise one of the largest families of insect genes so that future studies of the functional importance in cuticle morphogenesis of many other CPs, particularly ones belonging to different subfamilies, is of great interest.

6.3 Interactions and Functions of Pigments in Insect Ecology

The surfaces of insects show various types of colorations, which include structural colors and colors with versatile pigments. The structural colors are the results of optical effects by fine structures of cuticle surfaces (Seago et al. 2009). In addition, insect cuticles contain various types of substances that are responsible for

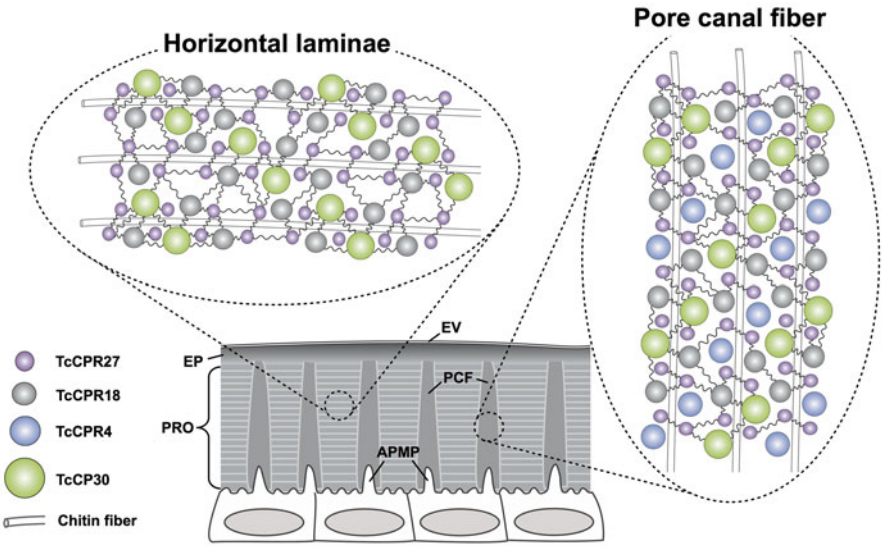

Fig. 6.6 Proposed cuticular proteins cross-linking in rigid cuticle of *T. castaneum*. Rigid adult cuticle is composed of three functional layers, envelope (EV), epicuticle (EP) and procuticle (PRO) in the 5 days-old adult of *T. castaneum*. Chitin and cuticular proteins are major components of a large number of horizontal laminae and vertical pore canal fibers in the procuticle. Major cuticular proteins, TcCPR27, TcCPR18 and TcCP30, are localized in both laminae and pore canal fibers, whereas TcCPR4 is predominantly localized in pore canal fibers (Mun et al. 2015; Noh et al. 2014, 2015b). TcCP30 undergoes cross-linking with TcCPR27 and TcCPR18, but not to TcCPR4 in rigid cuticle (Mun et al. 2015). Pigments are primarily localized in the procuticle

characteristic colors. Sometimes, there are correlations between color (pigment) patterns and the local shape of cuticle. For example, the *Junonia* butterfly wing eyespot pattern correlates with the thickness pattern of the underlying cuticle (Taira and Otaki 2016). With pigments, insects can create a variety of body color patterns that contribute to increased survival rates through camouflage, warning or mimicry and to accelerated sexual selection. The biological significance of body colors produced through tyrosine metabolism has been investigated as one major research area in many insect species. This section focuses mainly on the changes in the extent of melanization that are associated with environmental conditions.

During development, insects often change their body colors for better adaptation to the surroundings. As one example, the molecular mechanisms of body color transition are well described in *P. xuthus* (Futahashi and Fujiwara 2005, 2008). Until the fourth instar, the larvae show a pattern with white and brown colors that is thought to mimic the fecal material of birds. In contrast, during the molt to the fifth (last) instar, the pattern drastically changes to a greenish coloration that is cryptic for leaves. For this transition of body color pattern, the concentration of juvenile hormones (JH) is the critical factor (see Sect. 6.4). This transition in *P. xuthus* is an example of events that occur during the normal developmental process. Similarly, polyphenic phenotypes occur through changes in gene expressions that are regu-

lated by hormones (JH, ecdysteroid, peptidic factors) through modulations in the status of epigenetic modifications and metabolic pathways. The body color pattern is one polyphenic trait that can be modified by environmental cues. Locusts have two phases, a "solitaria phase" in low density of individuals and a "gregaria phase" under the opposite situation, each of which is characterized by a specific metabolism, behavior and body appearances. In the transition from solitary to gregarial phase, the greenish body is changed to blackish. By HPLC isolation and bioassay experiments with the albino mutant of *L. migratoria*, the peptide corazonin was identified from both *L. migratoria* and *S. gregaria* as the hormone that induces melanin synthesis (Tawfik et al. 1999; Tanaka 2000; Predel et al. 2007). Corazonin has an N-terminal pyroglutamate and C-terminal amidation, and the sequence is homologous to the human melanocyte-stimulating hormone α-MSH. Corazonins from the locust have a histidine residue at the seventh position instead of an arginine in corazonin from the cockroach, *P. americana*, and the peptide was identified as a heart-stimulating hormone in *P. americana* (Veenstra 1989, 1991). Corazonin induces not only melanogenesis, but also morphological changes (Tanaka et al. 2002; Maeno et al. 2004). It has been shown in *S. gregaria* that in a crowded situation, the signal of mechanoreception on the hind legs is transduced to the central nervous system (CNS). Then neuromodulation of the CNS leads to behavioral changes from solitary to the gregarious type. Either mechanoreception at the hind legs or a combination of sight and odor cues induce a pulse of serotonin in the metathoracic ganglion, and this is both necessary and sufficient for induction of behavioral changes for gregarization in *S. gregaria* (Simpson et al. 2011). The eggs laid by gregarious females have a tendency to be larger than those laid by solitary females and the proportion of "green hatching" from the larger eggs is lower (Tanaka and Maeno 2010).

Temperature appears to cause adaptive changes in the coloration pattern. The larval body of the five-spotted hawkmoth, *Manduca quinquemaculata*, shows two temperature-dependent phenotypes. Larvae developed at 20 °C have a blackened color, but those developed at 28 °C have a greenish color (Hudson 1966). The dark body color can be adaptive to obtain heat by absorbing sunlight in a low temperature condition, although the black coloration is thought to be in a trade-off relationship with the cryptic green color in leaves. In the related species, *M. sexta*, the *black* mutant shows similar characteristics. At normal temperature (20–28 °C) the body color is black, and low JH titer is responsible for this phenotype. In contrast, heat shock during the sensitive period in the fourth instar elevates the JH titer and suppresses *DDC* expression during the molt. The body color of the next instar is changed to green (Suzuki and Nijhout 2006). Such experiments to decipher the effect of temperature shifts have been performed since the nineteenth century. In many cases, a high-temperature shock induced phenotypes found in warm climates, and *vice versa* (Merrifield 1890, 1893). In a nymphalid butterfly, *Junonia orithya*, a cold shock during the pupal period shows polyphenic wing color patterns that are induced by a cold-shock hormone (Mahdi et al. 2010). Although such a correlation has been elusive, the cold-shocked individuals show an increase in dopamine content and a tendency to produce a darker phenotype. In *D. melanogaster*, growth

at a lower temperature induces a darker abdomen in adult females (David et al. 1990), which is associated with modulations of *TH* and *ebony* expressions (Gibert et al. 2007). This observation is consistent with the geographic distributions of darker variations (David et al. 1985; Gibert et al. 1996, 1998; Takahashi and Takano-Shimizu 2011; Telonis-Scott et al. 2011), indicating that this plasticity is an adaptive trait. The relationship between high latitudes (low temperature and weak sunlight) and darker body color has been reported in many cases (reviewed by True 2003; Rajpurohit and Nedved 2013; Takahashi 2013). The recent global warming trend has affected greatly the distribution of European insects (Zeuss et al. 2014). Based on the data obtained from a study of 473 European butterflies and dragonflies, it was found that dark and light colored insects are associated with cool and warm climates, respectively, and that the average darkness of the insects has decreased during the last century.

6.4 Hormonal Regulation of Cuticle Tanning

How does hormonal regulation lead to variations in pigmentation of the insect exoskeleton? Pigmentation development involves activating agents such as hormones that regulate the process, positioning agents that generate pigments in space and time, and the biochemical synthesis of pigments (Wittkopp and Beldade 2009). Genes in these steps are referred to as "regulating", "patterning" and "effector" genes, respectively. Regulating genes modulate patterning genes that determine the distribution of pigments by directly or indirectly activating expression of effector genes that encode the enzymes and co-factors required for pigment biosynthesis.

There are two main classes of insect hormones, the hormones produced by epithelial glands belonging to the ecdysteroids and juvenile hormones (JH) and also the neuropeptide hormones produced by neurosecretory cells. A working hypothesis is that the pigment biochemical module is regulated primarily by ecdysteroids such as 20-hydroxyecdysone (20HE) and JH as well as transcription factors expressed in a marking-specific pattern. Neuropeptide hormones such as bursicon also play regulatory roles in pigment metabolism.

Cuticular pigmentation in *P. xuthus* is caused by exposure to ecdysteroid and its subsequent removal (Futahashi and Fujiwara 2007). For instance, the larval marking eyespot pigmentation and differences in pigmentation timing appear to be regulated by ecdysteroid-inducible transcription factors that modulate melanin-synthesis genes. In this species, stage-specific larval marking color patterns are determined by a two-phase melanin-related gene prepatterning process (Futahashi et al. 2010). Two secreted protein genes, *yellow-y* and *laccase 2*, correlated with the black markings are expressed in the middle of the molting period (phase 1). In contrast, five epidermal proteins genes, *TH*, *DDC*, *GTPCH I* (*GTP cyclohydrolase I*), *tan* and *ebony*, associated with black or reddish-brown markings are expressed in the latter half of the molting period (phase 2). In addition, topical application of ecdysteroids prevents the induction of *TH*, *DDC* and *ebony* expression as well as maintains

yellow expression in the later molting stage, suggesting that the genes expressed in phase 1 require a high ecdysteroid titer, whereas the expression of the genes in phase 2 are induced by 20HE removal. Similar spatial and temporal expression profiles of most corresponding genes are observed in two other *Papilio* species with two additional genes, *bilin-binding protein* (*BBP*) and a *yellow-related gene* (*YRG*), whose combinations contribute larval blue, yellow and green colorations (Shirataki et al. 2010).

Two transcription factors, *spalt* and ecdysteroid signal-related *E75*, are genes expressed in the larval black eyespot markings in *Papilio* (Futahashi et al. 2012). The former expression correlated with the cryptic wing pattern (black marking) formation in several butterfly species, suggesting that it may be a positive regulator for melanin synthesis in both larvae and adult butterflies. The expression pattern of *E75* induced by ecdysteroids (Hiruma and Riddiford 2009) is correlated with the eyespot pigmentation pattern, which is similar to that of the *yellow* gene. In *M. sexta*, *DDC* expression is regulated by *E75* in response to exposure to ecdysteroids followed by its removal (Hiruma and Riddiford 1990, 2009). In *B. mori*, *tan* and *laccase 2* genes, which are also marking-specific (Futahashi et al. 2010), are apparently regulated by several ecdysteroid-inducible transcription factors including *ecdysone receptor*, *E75*, *hormone receptor 3* and *ftz transcription factor 1* (Hiruma and Riddiford 2009). These results suggested that *E75* is a strong candidate mediator of the hormone-dependent coordination of larval pigment pattern formation by regulating several black marking genes. Transcription factors such *as optomotor-blind* (*omb*) or *bric a brac* (*bab*) may also regulate patterning in insects (Wittkopp et al. 2003b). Color patterns in wings of butterflies are complex and may be the product of the co-option of developmental pathways, as exemplified by the eye development gene *optix*, which is correlated with wing patterns of *Heliconius* butterflies (Kronforst et al. 2012).

JH has been proposed to influence the expression pattern of 20HE-related genes (Riddiford 2008), and it induces the expression of genes associated with the mimetic pattern. In *P. xuthus*, a decrease in JH titer causes a switch to the cryptic pattern (Futahashi and Fujiwara 2008). Until the end of third instar larva, a high JH titer is maintained, but during the fourth instar larval stage, the titer declines. The low JH titer is the cause of the greenish cryptic pattern in the next instar, because injection of a JH analog (JH acid: JHA) at the early fourth instar larval stage suppresses the transition of body color pattern. With the injection of JHA, the spatial patterns of *TH* and *DDC* expressions in the cuticles of fourth instar larvae are maintained during the molt, and the mimicking brownish color pattern is maintained in the resulting fifth instar larvae. In addition to overall coloration, JH also regulates exoskeletal structures and pigment distribution at specific markings. In general, the hormonal condition and hormone sensitivity appear to be two of the main factors responsible for interspecific differences in larval color patterns in lepidopteran species such as the genus of *Papilio* (Shirataki et al. 2010).

The pupal color of the butterflies often shows dimorphic phenotypes, green and brown. The occurrence of the colors is dependent on the surrounding situations at the site for pupation. Optic signals from background color or tactile signals from the

surface of the pupation site (smooth or rough) have been studied as determinants for pupal colors (Hazel and West 1979; Hiraga 2005). Seasonal factors (photoperiod, temperature or humidity) can also have an influence on the pupal color determination (Hazel and West 1979, 1983; Sims and Shapiro 1984; Smith 1978, 1980). In the nymphalid butterfly, *Inachis io*, the browning of the pupa is controlled by the neuropeptide, pupal melanization-reducing factor (PMRF), that is released from the brain into hemolymph (Bückmann and Maisch 1987). In *P. xuthus*, the pupal color is controlled by pupal cuticle melanizing hormone (PCMH) and orange pupa inducing factor (OPIF; Yamanaka et al. 1999, 2004, 2006). Blackening of body color occurs in larvae of the armyworm, *P. separata*. In crowded conditions, "melanization and reddish coloration hormone" (MRCH) known for its FxPRL-amide structure at the C-terminus is released into the hemolymph to increase melanization of the cuticle. MRCH is homologous to the insulin-like growth factor (Matsumoto et al. 1981, 1986). In *P. separata* other physical stresses (cold shock or continuous mechanical stimuli) or parasitic wasp injection elevates the level of another type of peptide that shares sequence similarity with the epidermal growth factor (Hayakawa et al. 1995; Ohnishi 1954). This factor, growth-blocking peptide (GBP), is responsible for suppression of the increase in JH esterase levels after parasitization (Hayakawa 1991). GBP directly acts on epidermal cells to induce an elevation of cytoplasmic calcium ion concentration, which increases the levels of *TH* and *DDC* expression (Ninomiya and Hayakawa 2007).

At the enzyme level, the epidermal synthesis of a granular phenoloxidase (GPO) that catalyzes cuticular melanization of *M. sexta* larvae is inhibited by JH (Hiruma and Riddiford 1988). The absence of JH at the time of head capsule slippage during the last larval molt causes deposition of premelanin granules containing an inactive prophenoloxidase into the outer regions of the newly forming endocuticle. The decline of ecdysteroid titer allows activation of the proGPO and subsequent melanization (Curtis et al. 1984). Neuropeptide hormones such as MRCH, eclosion hormone, or extracts of pharate-larval subesophageal ganglia or corpora cardiaca-corpora allata complexes did not accelerate melanization. Dopamine, the precursor for melanin, is produced by the epidermis at the end of the molt, due to an increase in DOPA decarboxylase (DDC), which catalyzes dopamine production from DOPA (Hiruma and Riddiford 1993). The rise in DDC activity is controlled at the transcriptional level and dependent on the changing ecdysteroid titer during the molt as described above.

Bursicon (bur) known as "tanning hormone" is an insect heterodimeric neuropeptide hormone that is secreted from the central nervous system into the hemolymph, which initiates cuticle tanning as well as wing expansion after adult eclosion. Rickets (rk), the receptor for bur, is a member of the G protein-coupled receptor superfamily (Honegger et al. 2008; Luo et al. 2005; Mendive et al. 2005). In *D. melanogaster* larvae, Dmbur is expressed exclusively in crustacean cardioactive peptide-expressing neurons (Luan et al. 2006; Luo et al. 2005; Park et al. 2003). *D. melanogaster* mutants for *Dmbur* and the receptor mutant *Dmrk* showed deficiencies in both tanning and wing expansion (Baker and Truman 2002; Dewey et al. 2004). Eighty-seven genes in *D. melanogaster* were found to be sensitive to recombinant

bur stimulation (An et al. 2008). Thirty have no known function, but the others encode proteins with diverse functions, including cell signaling, gene transcription, DNA/RNA binding, ion trafficking, proteolysis-peptidolysis, metabolism, cytoskeleton formation, immune response and cell-adhesion. Huang et al. (2007) reported that loss of function of Bmbur caused a defect in wing expansion but no distinct tanning phenotype was observed in *B. mori* adults. Similarly, knockdown in the expression of genes coding for Tcbur and/or its receptor Tcrk in *T. castaneum* by injecting dsRNA into the pharate pupae caused a wrinkled elytra phenotype, but showed no effect on cuticle tanning. However, when Bai and Palli (2010) injected *Tcrk* dsRNA earlier in development (into young last instar larvae), pupal cuticle tanning was affected. A reduction in the expression of *laccase 2* in *Tcrk* RNAi beetles suggested that Tcrk influences pupal cuticle tanning by regulating the expression of *laccase 2* in *T. castaneum*. However, post-translational modifications such as phosphorylation may also play a role in regulation of cuticle tanning by the bursicon receptor (Honegger et al. 2008). TH is transiently activated during tanning by phosphorylation at a serine residue as a result of bursicon signaling (Davis et al. 2007). A novel bursicon-regulated gene in the housefly, *Musca domestica* (named md13379 because it is homologous with CG13379 in *D. melanogaster*), encodes a transcriptional regulator homologous to human ataxin-7-like3 and yeast sgf11, both of which encode a novel subunit of the Spt-Ada-Gcn5-acetyltransferase (An et al. 2009). Proteins related to md13379 may play a role in regulating the expression of bursicon-regulated genes involved in cuticle tanning.

6.5 Future Prospects and Concluding Remarks

Although tyrosine metabolism for insect cuticle tanning and the chemistry of pigment synthesis in general are still not well understood, past studies have demonstrated several physiologically and ecologically important roles in the tanning process. These findings suggest that the potential for development of novel control agents targeting insect cuticle tanning physiology. For example, after demonstration of specific gene down-regulation in several insect species including crop pests by orally administered dsRNA (Turner et al. 2006; Zhou et al. 2008; Tian et al. 2009; Walshe et al. 2009; Chen et al. 2010; Huvenne and Smagghe 2010; Zhang et al. 2010; Li et al. 2011; Bolognesi et al. 2012; Zhu et al. 2012; Miyata et al. 2014; Wan et al. 2014; Guo et al. 2015), RNAi based-pest management is now considered to be a promising new technology to control target pest insects with high species-specificity and environmentally friendly attributes. In addition, transgenic plants expressing dsRNAs for target insect genes impair growth and development (Baum et al. 2007; Mao et al. 2007; Pitino et al. 2011; Zha et al. 2011; Zhu et al. 2012; Kumar et al. 2013; Zhang et al. 2015). These results suggest that RNAi based-pest control could be a very useful alternative to chemical pesticides for suppression of economically damaging pest insects. Loss of function of many genes involved in the tyrosine-mediated cuticle tanning pathway caused not only alterations in color but also resulted in high mortality as described in

Sect. 6.2, indicating that the pathway is vital for insect development, growth and survival, making the genes potential targets for controlling economically important pest insects and animal/plant disease vectors.

The involvement of phenoloxidases in cuticle tanning is essential to insect survival (Dittmer and Kanost 2010) and laccases are potential phenoloxidase targets for insect control agents. One of the first compounds developed to adversely affect insect cuticle tanning is the sterically hindered phenol, 2,6-ditert-butyl-4-(2-phenylpropan-2-yl)-phenol (MON-0585, Semensi and Sugumaran 1986). In MON-0585-treated insects there was a reduced phenoloxidase activity as compared to controls as well as a significant reduction in the level of covalently bound catechols and malformations in the exoskeleton. Prasain et al. (2012) tested substituted phenolic compounds as substrates or inhibitors of laccase and also examined their effects on mosquito larval growth. Several compounds caused >90 % mortalities as mosquito larvicides at nM-μM concentrations. The treated larvae started to separate the larval cuticle, but they failed to shed it. Also there was very little synthesis of new pupal cuticle by the treated larvae. Thus, some of the laccase-active compounds affected the development of the cuticle, which is a target that is different from the neurological targets of most currently utilized insecticides. However, the exact target and mechanism of action of these modified phenolic compounds *in vivo* still remain to be determined.

In biomimetic/entomomimetic science, an area of research involves the development of novel biomimetic materials with unique physical properties similar to those of the pigmented insect exoskeleton for use in biomedical or other technological devices. For example, a pigmented cuticle structure of the highly modified and tanned forewing elytron of beetles was investigated as a biomimetic model and its application was described (Dai and Yang 2010; Lomakin et al. 2011; Chen and Wu 2013; Chen et al. 2015a, b). Unlike other pigmented and rigid cuticles in different body regions of adult beetles, the elytron composes of dual cuticular layers (dorsal and ventral cuticles) connected by a large number of pillar-like fibrous structures called trabeculae. The unique structure of the elytron, for instance, has been applied as a model to develop novel integrated honeycomb structures that are lightweight with a high mechanical strength (Chen et al. 2012, 2015b).

Although substantial progress in studies of insect cuticle tanning metabolism has occurred in the past, there remain several unanswered questions that should be addressed in future studies. What are the exact chemical structures of the insect pigments? Structural models and templates have been offered for some of the pigments including eumelanin and pheomelanin (Solano 2014), but the exact structures have not been established. How do the pigments interact with other components for assembly of the supramolecular extracellular matrix known as the exoskeleton? How do the pigments interact covalently and non-covalently with other components in the cuticle including proteins, chitin, lipids, minerals and water? What is the contribution of the pigments to the mechanical properties of exoskeletons? Substantial research designed to answer these questions remains to be conducted in the future so that we will have a more complete understanding of

the chemistry, physiology and genetics of insect cuticle pigmentation and sclerotization.

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