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Herbivore Adaptations to Plant Cyanide Defenses

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Abstract

As plants are fixed to their habitat they produce specialized metabolites as chemical defenses to fight off herbivores. As an example, many plants produce cyanogenic glucosides and release toxic cyanide upon tissue damage (“cyanide bomb”). As a prerequisite for exploring cyanogenic plants as hosts, herbivores have evolved mechanisms to overcome cyanogenic defenses. Mammals metabolize cyanide to thiocyanate by rhodanases. In arthropods, both rhodanases and β -cyanoalanine synthases which transfer cyanide to cysteine contribute to cyanide detoxification. However, based on enzyme activity tests some arthropod species possess only one of these activities, and some possess both. Recently, cloning and characterization of first arthropod β -cyanoalanine synthases provided evidence for their involvement in cyanide detoxification. Phylogenetic analyses suggest that they have been recruited from microbial symbionts. Investigations with *Zygaena filipendulae* revealed that the avoidance of cyanide release is the primary mode of overcoming cyanide in this specialist. Some herbivores are able to sequester, *de novo* synthesize, and store cyanogenic glucosides for their defense and as nitrogen source. Thus, herbivores have evolved various mechanisms to counteract host plant cyanide defenses. These mechanisms are likely to have played a key role in the evolution of plant-herbivore interactions as well as in speciation and diversification of arthropods.

Keywords: cyanide detoxification, plant secondary/specialized metabolism, cyanogenic glucosides, β -cyanoalanine synthase, rhodanase

1. Introduction

Herbivores are a main threat for plants as their feeding destroys vegetative and generative parts of the plant, that is, organs needed for assimilation, nutrient storage and reproduction. In order to cut their losses, plants have developed physical and chemical defenses to

fight off herbivores and to survive in their ecosystem. Very effective means to defend against predators are provided by the so-called specialized (or “secondary”) metabolism, which is not required for growth and development, but for the plant’s interaction with its environment [1, 2]. Specialized metabolism is the source of diverse low molecular weight compounds such as alkaloids, terpenes, glucosinolates or cyanogenic glucosides, which are often specific to certain families or species. These compounds may repel the potential predator before contact or harm the herbivore upon ingestion. Defensive metabolites may have herbivore-specific effects or be universally toxic. In the latter case, they need to be stored in an inactive or nontoxic form in the plant to avoid self-intoxication.



Figure 1. Sources of cyanogenesis upon herbivory. Exemplary precursors, intermediates and reactions leading to the liberation of (hydrogen)cyanide from the four main pathways are shown. (A) Cyanogenic glucoside hydrolysis, (B) metabolism of aromatic glucosinolates in the herbivore *P. rapae*, (C) final step of the ethylene biosynthesis pathway, (D) cyanolipid hydrolysis. Plant families and groups forming the precursor compounds are shown in gray. Reactions involving plants are illustrated by blue arrows, reactions involving herbivore proteins by red arrows and spontaneous reactions by black arrows. For references and details, see the main text.

Chemical defense through cyanide is widespread in the plant kingdom. As a universal respiration toxin, cyanide is not accumulated in free form in plants but released from cyanogenic precursors upon tissue damage (“cyanide bomb”), in the course of metabolic reactions in intact plant tissue or upon ingestion by herbivores (**Figure 1**). The acute and universal toxicity of cyanide in combination with its frequent occurrence in the plant kingdom calls for efficient cyanide detoxification mechanisms in herbivores. As soon as cyanide is liberated upon ingestion of cyanogenic plant material, an enzymatic detoxification is vital for the protection of the herbivore’s cellular metabolism. Although diverse enzymatically catalyzed reactions for the detoxification of cyanide have been described in microorganisms [3], only two main pathways of cyanide detoxification are present in higher animals. These are on the one hand the rhodanese or 3-mercaptopyruvate sulfurtransferase-catalyzed transfer of sulfur from a donor substrate to cyanide, leading to the formation of thiocyanate, and, on the other hand, the β -cyanoalanine synthase-catalyzed substitution of cysteine’s sulfhydryl group by cyanide, leading to the formation of β -cyanoalanine.

An efficient way to minimize the risk of cyanide poisoning is to prevent its formation. Therefore, herbivorous arthropods that colonize plants with high cyanide potential often possess specialized adaptations, which allow them to avoid cyanide release upon ingestion of plant material. This chapter introduces cyanide as a ubiquitous plant-produced compound and summarizes the present understanding of cyanide detoxification pathways and the involved enzymes as well as the current knowledge on cyanide avoidance mechanisms in herbivores with a special focus on arthropods. As certain arthropod species are able to synthesize cyanogenic compounds themselves and/or to sequester cyanogenic compounds from their food plants, we also discuss cyanogenesis in herbivores from an ecological and evolutionary perspective.

2. Sources of cyanide exposure

The most common storage form of cyanide in plants is cyanogenic glucosides, which are potent antiherbivore defenses with an additional function as nitrogen storage compounds [4–8]. The intact glucosides are water-soluble and nontoxic compounds, but hydrolysis catalyzed by β -glucosidases liberates the cyanohydrins (α -hydroxynitriles), which, spontaneously or under catalysis by α -hydroxynitrilases, release the hydrogen cyanide next to aldehydes or ketones (**Figure 1A**) [9]. Their broad distribution among more than 2650 plant species from the pteridophytes, gymnosperms and angiosperms [9, 10] may be explained by their biosynthetic origin. Cyanogenic glucosides are biosynthesized through oxidation of common aliphatic and aromatic amino acids by members of the wide-ranging cytochrome P450 family with oximes and cyanohydrins as intermediates and subsequent *O*-glycosylation [11]. Although cyanogenic glucosides are widespread within the plant kingdom and their biosynthetic enzymes are ancient [12], <30 different structures of plant-derived cyanogenic glucosides have been described. Among those, linamarin, lotaustralin and dhurrin (**Figure 1A**), derived from the amino acids valine, isoleucine and tyrosine, respectively, are the most common glucosides. Activation of the glucosides under cyanide liberation happens upon herbivore attack when the glucosidic bond is hydrolyzed by endogenous plant β -glucosidases or digestive enzymes

of the herbivore (**Figure 1A**). Plants possess β -glucosidases with high specificity toward their own defensive cyanogenic glucosides [13].

Some cyanogenic glucosides have not only been found in plants, but are sequestered and even biosynthesized *de novo* in herbivores and detritivores from the Arthropoda [14, 15]. As a prominent example, larvae of *Zygaena filipendulae* (Lepidoptera: Zygaenidae) which are specialist herbivores on the cyanogenic bird's-foot trefoil *Lotus corniculatus* are able to sequester cyanogenic glucosides from the host plant and to synthesize them *de novo* by a pathway that has evolved independently from that in plants (see below) [16, 17]. Both the plant and the insect benefit from inherent cyanogenic glucosides as a chemical defense and as nutrient storage compounds [4, 6]. Thus, the *Zygaena*-*Lotus* association does not only illustrate the coevolutionary arms race between plants and their herbivores but also provides an example of convergent pathway evolution [16, 18, 19]. Cyanide intoxication from the diet is also relevant to humans as some major foodstuffs, especially in tropical regions, contain cyanogenic glucosides. In particular, cassava tubers, green sorghum leaves, white clover foliage and lima beans have been reported as potential sources of cyanide to man. Nevertheless, traditional food preparation and selective breeding to decrease cyanide levels lower the risk of accidental cyanide poisoning.

A further class of plant secondary compounds carrying a cyanide group is the cyanolipids, a group of lipids possessing a branched five carbon skeleton with a nitrile group [20]. Cyanolipids occur in the seed oil of diverse species of the Sapindaceae [21]. They are cyanohydrin esters, that is, they possess an esterified hydroxyl group in α -position to the nitrile moiety and will form unstable α -hydroxynitriles upon spontaneous or lipase-catalyzed ester hydrolysis (**Figure 1D**) [22]. As described above, α -hydroxynitriles are a source of cyanide as they readily decompose either spontaneously or enzymatically catalyzed.

Cyanide may also be liberated upon metabolism of another group of specialized metabolites, the glucosinolates, inside the herbivore by the consecutive action of plant- and herbivore-expressed enzymes. Glucosinolates are amino acid-derived thioglucosides with a sulfated aldoxime core and a variable side chain [23] (**Figure 1B**). They are part of the glucosinolate-myrosinase system or "mustard oil bomb," a constitutive defense mechanism common to all families of the Brassicales. The products arising from glucosinolate hydrolysis have manifold effects on herbivores feeding on Brassicales plants, including general deterrence and toxicity, but may also be perceived by specialist herbivores and their parasitoids as host identification cues [24]. The primary defense compounds derived from this system are the isothiocyanates which result from rearrangement of the aglucone formed upon hydrolysis by co-occurring thioglucosidases (myrosinases) when tissue is disrupted [23, 25]. Besides isothiocyanates, other products such as nitriles, epithionitriles and organic thiocyanate can also be formed depending on the structure of the glucosinolate side chain and the presence of additional plant-expressed proteins, the so-called specifier proteins [26–29]. Cyanide release from a glucosinolate-derived nitrile has been demonstrated to occur in larvae of *Pieris rapae* (Lepidoptera: Pieridae) which are specialized feeders on glucosinolate-containing plants [30] (**Figure 1B**). Larvae of *P. rapae* and other glucosinolate-feeding Pieridae produce a gut nitrile-specifier protein to overcome the glucosinolate defense of their host plants [31, 32]. Upon ingestion of glucosinolate-containing plant material, this protein redirects glucosino-

late hydrolysis catalyzed by plant myrosinases entirely toward nitriles (instead of isothiocyanates) which are excreted or further metabolized [33, 34] (**Figure 1B**). Phenylacetoneitrile (derived from benzylglucosinolate) undergoes α -hydroxylation by microsomal *P. rapae* enzymes yielding a cyanohydrin which is subsequently decomposed to cyanide and an aldehyde [30] (**Figure 1B**). Despite liberation of one cyanide molecule per molecule of ingested benzylglucosinolate, the larvae feed on benzylglucosinolate-containing plants without ill effects indicating adaptation to this toxin [30]. Expression of a gut nitrile-specifier protein appears to be confined to Pieridae species, while glucosinolate-feeding species of other families overcome the glucosinolate-myrosinase system by other means [32, 35]. If herbivores devoid of a gut nitrile-specifier protein may also encounter cyanide liberated from glucosinolate breakdown products is presently uncertain. However, cyanide release from glucosinolate metabolites in homogenates of *Alliaria petiolata* (Brassicaceae) [36], nitrile formation upon glucosinolate breakdown in plant homogenates [27, 28, 37] and the likely ability of herbivore phase-I-detoxification enzymes to hydroxylate these nitriles to cyanohydrins [38] indicates that herbivores outside the Pieridae are likely exposed occasionally to cyanide when feeding on glucosinolate-producing plants.

Apart from the accumulation of cyanogenic precursors as part of specialized metabolism for defense against herbivores, plants from all families generate cyanide as a by-product during the formation of ethylene, a ubiquitous plant hormone. In the last step of ethylene biosynthesis, the oxidation of 1-aminocyclopropane-1-carboxylic acid to ethylene, one mole of cyanide is liberated per mole of ethylene formed (**Figure 1C**) [39]. Although the steady-state concentration of cyanide from this pathway is normally kept at a low level of 0.2 μ M by action of cyanide detoxifying enzymes [40], this demonstrates the ubiquitous occurrence of cyanide in the feed of herbivores.

Taken together, cyanide is universally present in plants and herbivores are frequently confronted with this toxin through their diet. Thus, safe handling of cyanide is a necessary prerequisite for herbivory by both specialists feeding exclusively on cyanide-defended plants and generalists with occasional cyanide ingestion. Nevertheless, a varying cyanide content in the host plant seems to influence generalist herbivory more severely than specialist feeding indicating the existence of efficient adaptations to deal with this toxin [41].

3. Cyanide toxicity

Uptake of the small and simple ion cyanide has tremendous effects on the metabolism of all aerobic cells, resulting from its high reactivity and efficient binding to various proteins of cellular respiration and regulation. The main reason for its acute and universal toxicity is the formation of stable complexes between cyanide and the Fe^{3+} -ion of heme a_3 of cytochrome c oxidase, one of the electron carriers in the respiratory chain. Cyanide binds to cytochrome c oxidase and acts as a noncompetitive inhibitor of cytochrome c. This stops electron transfer, leading to termination of the respiratory chain and the citric acid cycle due to a shortage of the electron acceptor NAD^+ [42]. The resulting lack of ATP is detrimental to the cell. As a con-

sequence, glycolysis, the alternative, but inefficient pathway of ATP generation, is accelerated in combination with lactic acid fermentation for regeneration of NAD^+ . In humans, metabolic acidosis resulting from high lactic acid levels is responsible for most of the symptoms of cyanide intoxication [43]. Besides the Fe^{3+} of cytochrome c oxidase cyanide binds metal ions of various metalloenzymes, in particular molybdoenzymes, and forms Schiff base intermediates with pyridoxal phosphate-dependent enzymes causing an efficient inhibition of a wide range of metabolic reactions and regulatory processes in the cell [44].

In vertebrates, cyanide does not only influence cellular metabolism but also diverse physiological processes. By binding to chemoreceptors, cyanide causes vasoconstriction of main arteries which may lead to cardiac shock or pulmonary edema [43, 45]. In addition, cyanide may increase neurotransmitter release by influencing calcium channels in neural cell membranes [46]. Even sublethal doses of cyanide may harm the brain of mammals by altering the membrane lipid peroxidation and the response of antioxidant enzymes [47].

Several studies performed by Edwin J. Bond in the 1960s on the beetle *Sitophilus granarius* have investigated the effect of cyanide on insects as discussed by Page and Lubatti [48]. The studies showed that consumption of cyanide even at low doses results in immediate paralysis of the animals by reduced respiration [48, 49]. Lethal effects are not only due to cytochrome c oxidase inhibition but also result from increased proteolysis and binding of cyanide to intermediates of glycolysis. In contrast to mammals, where supplementation of oxygen is an efficient treatment of cyanide poisoning, the administration of oxygen to cyanide-exposed insects amplifies the ill effects of cyanide, probably due to the accumulation of promptly formed peroxides and acidosis-causing citrate and pyruvate. In contrast, oxygen exclusion allows the animals to recover from the poisoning [50, 51].

Thus, the mode of action of the poison cyanide is complex, and the lethal effects differ between species. Nevertheless, cyanide is one of the most potent toxins and an efficient and universal weapon of plants against herbivore foraging.

4. Cyanide detoxification enzymes

4.1. Sulfur transferases

Sulfur transferases such as the rhodanese (thiosulfate:cyanidesulfurtransferase, EC 2.8.1.1; see **Figure 2A**), and its close relative, the 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2), are enzymes described in plants, fungi, bacteria and a wide range of animals including snails, insects, fish and mammals (see **Figure 3**) [52–58]. Enzymatic formation of thiocyanate, the so-called rhodanide, was first described in 1933 using vertebrate tissues as discussed by Lewis [59]. Rhodanases from mammals have been investigated most thoroughly and most insight has been gained from the examination of human and bovine liver rhodanases [60, 61]. These two enzymes served to uncover the first protein structure of a rhodanese which revealed two similarly folded “rhodanese domains” [62]. In contrast to the highly similar tertiary structure, the two domains differ strongly in their amino acid sequences in agreement with their diver-

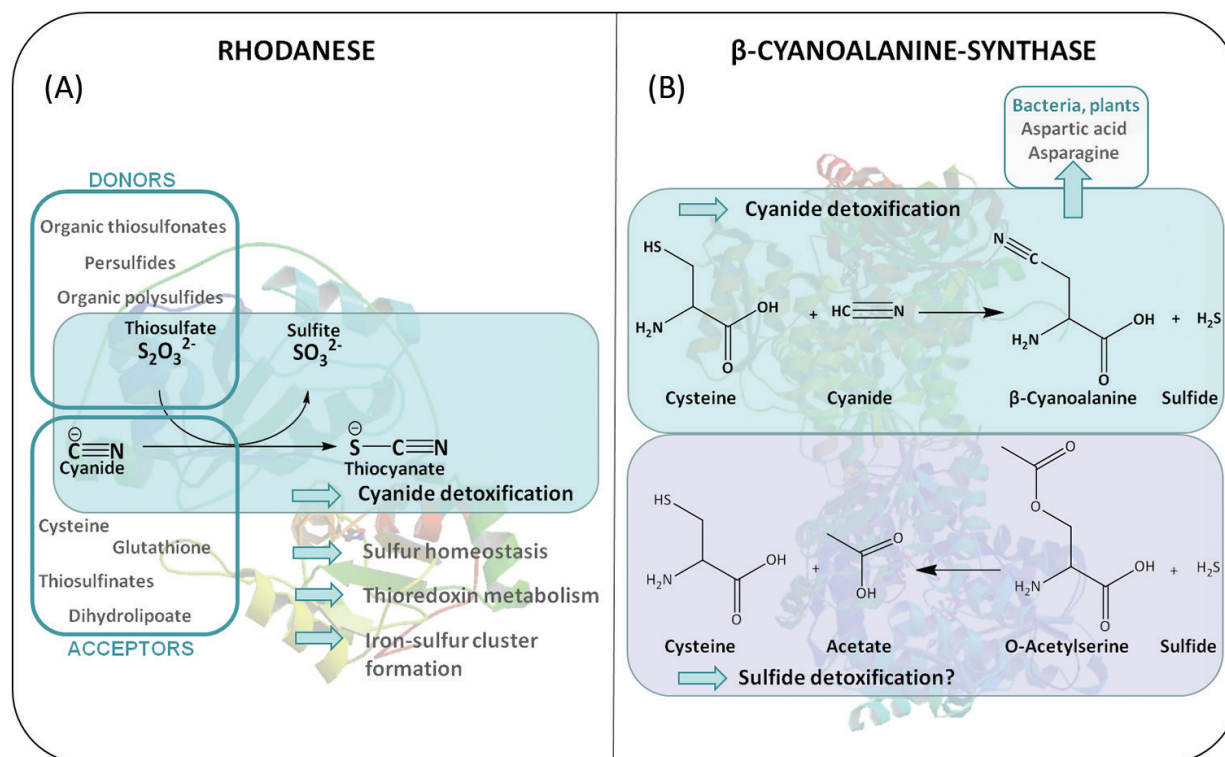


Figure 2. Main cyanide detoxification enzymes. Shown are reactions in which cyanide conversion is catalyzed by rhodanese (A—background: bovine liver rhodanese PDB 1RHD) or β -cyanoalanine synthase (B—background: soy bean β -cyanoalanine synthase PDB 3VBE). Additional rhodanese substrates are also shown (A). The *O*-acetylserine thiol lyase reaction is shown in the lower panel of B. Block arrows indicate possible alternative and additional roles of the enzymes.

gent functions as C-terminal catalytical and N-terminal regulatory domains [62]. At the level of primary structure, rhodanases from different organisms show little similarity apart from two conserved, 11–13 amino acids long “signature” regions at the N- and C-termini which are also present in distantly related proteins of the rhodanase superfamily such as *cdc25* phosphatase and heat shock proteins [63]. This low identity at the amino acid level and the involvement of single rhodanase domains in aberrant proteins make homology-based identification of rhodanases from further species difficult. To specify the sulfur transferases involved in cyanide detoxification among the members of the diverse superfamily, the tertiary structure has to be taken into account, classifying the true rhodanases as tandem domain thiosulfate:cyanide sulfurtransferases [63]. This group also comprises the 3-mercaptopyruvate sulfurtransferases. Both types of enzymes have distinct substrate and product spectra, but are yet interconvertible by few amino acid substitutions [64].

Rhodanases do not only accept thiosulfate as sulfur donor, but all sulfane anions such as organic sulfanes and persulfides [55, 65]. Next to cyanide, the sulfur atom may be accepted by other thiophilic substrates such as the amino acids cysteine and glutathione [65]. The kinetic mechanism of rhodanase was uncovered with its classical substrates cyanide and thiosulfate by Westley and coworkers [60, 66]. In a ping-pong reaction, the sulfane sulfur atom is abstracted from the donor substrate thiosulfate and bound to a cysteine residue in the active site of rhodanase [62]. This is followed by entrance of the acceptor substrate cyanide into

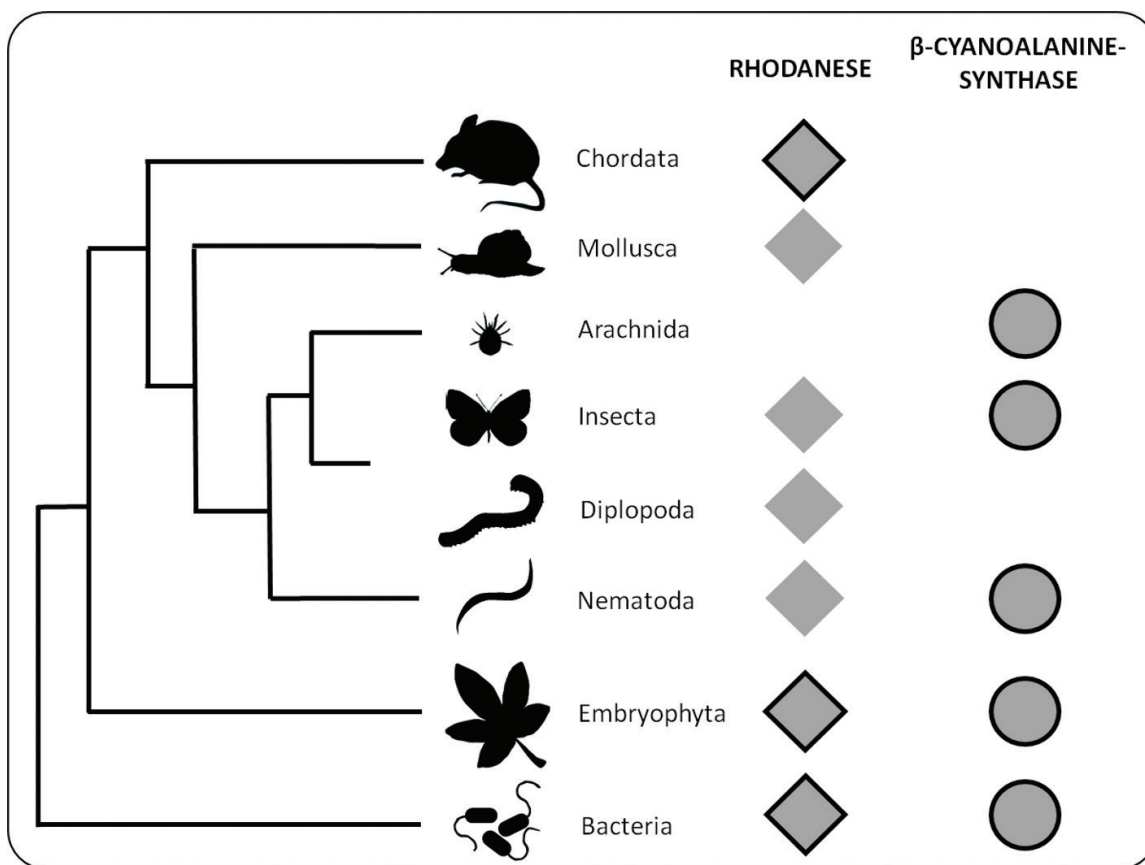


Figure 3. Occurrence of cyanide detoxification enzymes in living organisms. Selected domains, subkingdoms, phyla and classes are shown in a schematic representation of their phylogenetic relationship. Groups in which enzyme activity has been detected are labeled with a gray area (square for rhodanese, circle for β -cyanoalanine synthase). The area is surrounded by black line if sequences of the corresponding enzymes or their genes have been elucidated. Metabolite data also proved β -cyanoalanine synthase activity in Diplopoda, where activity assays have not been performed to our knowledge. Rhodanese seems to be an ubiquitous enzyme, although no sequence data are available from Arthropoda or Mollusca. In contrast, β -cyanoalanine synthase was detected primarily in plants, bacteria and Arthropoda.

the active site and transfer of the sulfur atom [60, 66]. In contrast, the reaction of 3-mercaptopyruvate sulfurtransferase (which can also convert cyanide to thiocyanate [67]) follows a sequential mechanism with formation of a ternary complex composed of the enzyme and both substrates (3-mercaptopyruvate and cyanide) [68, 69].

A main function of the rhodanases in cyanide detoxification is in agreement with their subcellular localization. Rhodanase activity is predominantly detected in the mitochondria, the site of cellular respiration with the cyanide-susceptible cytochrome c oxidase [70, 71]. Nevertheless, for 3-mercaptopyruvate sulfurtransferase and rhodanase of some species, an additional localization in the cytosolic fraction has been described [55, 71, 72]. The cytosolic enzymes may serve to reduce cyanide levels in this compartment whose components (glycolysis intermediates, proteins) may also be affected by cyanide poisoning (see above) [48]. The reaction product of rhodanase-catalyzed cyanide detoxification, thiocyanate, possesses a toxic potential toward mitochondria which could be an additional reason why rhodanase isoforms are also localized in the cytosol in several species.

Although a major role of rhodanases in cyanide detoxification seems likely based on the present knowledge, cyanide detoxification might not be their exclusive physiological function. The ubiquitous occurrence of rhodanases in organisms and tissues with no obvious cyanide exposure as well as the low physiological concentration of their substrate thiosulfate in the mitochondria has fueled doubts about their main role in cyanide detoxification [73]. In support of a major role in cyanide detoxification in mammals, rhodanase activity is inducible in rats by exposition to cyanide or supplementation with thiosulfate [74, 75]. *In vivo*, sulfane substrates may be provided by the action of an enzyme involved in cysteine metabolism in animals, cystathionine- γ -lyase [76]. Further and alternative functions of rhodanases have been discussed. As plant homologs have been shown *in vitro* to supply sulfide for iron sulfur clusters of the electron transport chain, this has been suggested as a general function of rhodanases from other taxa [73, 77]. Alternatively, a general role of rhodanases in the regulation of sulfur homeostasis has been proposed based on their low substrate specificity. The *in vivo* function of 3-mercaptopyruvate sulfurtransferases in cyanide detoxification has been questioned not least because addition of 3-mercaptopyruvate led to an antidote effect only in some mammal species [78]. Additionally, it has been difficult to separate their possible direct and indirect contributions to cyanide detoxification *in vivo*, as 3-mercaptopyruvate sulfurtransferase can also donate sulfur for the formation of the rhodanase substrate thiosulfate [79].

4.2. β -Cyanoalanine synthase

The main enzyme of cyanide detoxification in plants and many bacteria, β -cyanoalanine synthase (EC 4.4.1.9, **Figures 2B** and **3**), belongs to the family of β -substituted alanine synthases sharing the cofactor pyridoxal-5'-phosphate and a uniform fold [80, 81]. This family also comprises *O*-acetylserine thiol lyase and cystathionine- β -synthase which occur in bacteria, plants, fungi and animals and are involved in cysteine biosynthesis from *O*-acetylserine or serine and homocysteine, respectively [80, 82]. The amino acid sequence of these enzymes and their tertiary structure, the so-called fold II of the pyridoxal-5'-phosphate-dependent enzymes, are highly conserved. However, the members of the protein family vary in the quaternary structure, as mono-, di-, tetra- and oligomers have been reported, although dimers are most predominant [80, 83, 84]. Some members of the family can catalyze several of the above-mentioned reactions making a classification as β -cyanoalanine synthase or *O*-acetylserine thiol lyase difficult. Thus, plant enzymes are mainly assigned to an enzyme clade based on a comparison of kinetic characteristics for the diverse reactions catalyzed and their subcellular localization [80]. The only phyla of multicellular animals in which β -cyanoalanine synthase activity has been demonstrated up to now are Nematoda and Arthropoda (see **Figure 3**) [14, 85–87]. Only few animal β -substituted alanine synthases with β -cyanoalanine synthase activity have been identified to date (see below). As the protein family's name indicates, β -substituted alanine synthases catalyze a substitution or elimination reaction at the β -carbon of proteinogenic and nonproteinogenic amino acids. Their reaction mechanism has been examined mainly on the basis of prokaryotic and plant *O*-acetylserine thiol lyases [88–90]. All examined β -substituted alanine synthases share a common fold and perform a similar conformational change upon substrate binding to close the active site for catalysis [90]. In the active site, a Schiff base binding between the cofactor pyridoxal-5'-

phosphate and a catalytic lysine residue awaits the substrate amino acid [90]. Upon entrance of the substrate, pyridoxal-5'-phosphate is transferred from the lysine residue to the α -amino group of the substrate as an external Schiff base. Lysine's free amino group now acts as a base catalyst for the deprotonation of the α -carbon, inducing the α,β -elimination of sulfide, acetate or water from the substrate amino acid [88, 90]. The formed α -aminoacrylate intermediate is shared by all members of the β -substituted alanine synthase family regardless of the catalyzed reaction [88]. After formation of this intermediate, the second substrate enters the active site. It attacks the amino acid's side chain nucleophilically. Facilitated by acid catalysis by the protonated lysine residue, a bond between the β -carbon and the second substrate is formed. The newly formed amino acid product can then be released from the active site under reformation of the internal Schiff base between pyridoxal-5'-phosphate and lysine [90]. Because of high conservation in the reaction mechanism of different β -substituted alanine synthases, an *O*-acetylserine thiol lyase and a β -cyanoalanine synthase from soybean were analyzed for their substrate and product spectra by mutagenesis studies [90]. This showed that the exchange of only three amino acids can switch an *O*-acetylserine thiol lyase to a β -cyanoalanine synthase, illustrating their close relationship and giving insight into the evolution of β -substituted alanine synthases [90].

Next to amino acid biosynthesis, β -substituted alanine synthases are involved in cellular sulfur and redox homeostasis [84, 91–93]. Cyanide detoxification by these enzymes is mainly catalyzed by β -cyanoalanine synthases, but *O*-acetylserine thiol lyases can also bind the toxic ion to either cysteine or *O*-acetylserine [81]. According to enzyme activity assays, β -cyanoalanine synthases from plants and animals are mainly localized in the mitochondria [86, 94]. However, the recently identified arthropod β -cyanoalanine synthases do not seem to possess a mitochondrial signal sequence [87, 95]. Cytosolic expression might be beneficial as cyanide detoxification by β -cyanoalanine synthases leads to the formation of equimolar amounts of sulfide which itself is an inhibitor of cytochrome c oxidase. If released in the cytosol (instead of the mitochondria), sulfide can immediately be captured by the cytosolic *O*-acetylserine thiol lyases. In plants, experiments with T-DNA insertion mutants have shown that cytosolic *O*-acetylserine thiol lyases are essential for safe and efficient disposal of cyanide [92].

β -Cyanoalanine itself may also exert harmful effects. It has been identified as a neurotoxin and can also be lethal to plants [96, 97]. In order to protect themselves from poisoning with β -cyanoalanine and to minimize costs, plants and microorganisms are able to turn over β -cyanoalanine by nitrile hydratases and nitrilases [98]. Nitrile hydratases catalyze the addition of a water molecule to β -cyanoalanine leading to the formation of the proteinogenic amino acid asparagine. Nitrilases convert β -cyanoalanine to the proteinogenic amino acid aspartate by addition of two water molecules [99]. In addition, the conversion of β -cyanoalanine to asparagine with γ -glutamyl- β -cyanoalanine as an intermediate has been described for some plants [96]. Recycling of β -cyanoalanine has also been shown in some arthropod species (see below). Due to its neurotoxic effect, β -cyanoalanine stored in the defensive droplets of the cyanogenic lepidopteran species *Z. filipendulae* has also been discussed to directly act as a defensive compound for the protection of this herbivore against predators [100].

5. Cyanide detoxification strategies in herbivores

5.1. Cyanide detoxification in mammalian herbi- and omnivores

In mammals, rhodanese is generally believed to be the major enzyme for cyanide detoxification, while β -cyanoalanine synthase activity has not been detected in a mammal so far. A comparison of rhodanese activity between mammalian herbi-, omni- and carnivores shows highest activities in herbivores, especially in ruminants which feed on a broad range of plant material including plants with high cyanide potential [58]. In several mammalian species such as plant-feeding rabbits, rhodanese activity is ubiquitously distributed in the body with the highest activity in hepatocytes, the main detoxification site of e.g. xenobiotics [101, 102]. Rhodanese activity is also localized in the mammalian brain, where cyanide acts as a neuro-modulator [63].

5.2. Cyanide detoxification in invertebrates

Intensive research on mammalian rhodanases also raised the question whether these enzymes are involved in cyanide detoxification in other animals. Rhodanese activity is widely distributed in insects and occurs in snails (see **Figure 3**) [55, 103]. The level of activity is comparable to that of mammalian gut tissue [53]. However, activity levels are largely in the same range among herbivores which frequently or rarely encounter high cyanide levels. The basal rhodanese activity might be sufficient to capture dietary cyanide in herbivores regardless of the cyanide level in the diet. Alternatively, the uniform distribution of rhodanese activity among herbivores could indicate that arthropod rhodanases possess an additional function unrelated to cyanide detoxification. This would likely require other mechanisms of cyanide detoxification such as β -cyanoalanine synthase activity [103]. In agreement with this, β -cyanoalanine activity has been found to be broadly distributed in arthropod herbivores (see **Figure 3**) [86, 95, 104]. In support of the activity data, labeled β -cyanoalanine can be detected in arthropods after feeding of or exposition to isotopically labeled cyanide [30, 105]. Further support for the relevance of β -cyanoalanine synthases comes from experiments with several cyanide-forming lepidopteran species in whose defensive glands β -cyanoalanine and its hydration product asparagine were detected [106]. In millipedes, β -cyanoalanine synthase-catalyzed detoxification of cyanide and further metabolism of β -cyanoalanine to asparagine was demonstrated by studies with radiolabeled precursors [14, 107]. For insects, a similar utilization of cyanide for the formation of proteinogenic amino acids using β -cyanoalanine as an intermediate has been discussed [18], but has only been proven for one beetle by radioactive feeding experiments so far [108]. In the beetle, the radioactive label was recovered from a polypeptide rich in aspartate, the product of nitrilase-catalyzed conversion of β -cyanoalanine [108].

In order to estimate the relevance of rhodanese *vs.* β -cyanoalanine synthase for the *in vivo* detoxification, several studies compared the enzyme activities within one species upon cyanide induction or in related species with or without cyanide specialization [53, 86]. Most of these studies are more than 20 years old, and they cover only a very limited number of species. Nevertheless, for rhodanese no induction of activity upon cyanide feeding was observed although the occurrence in Diptera species parasitizing the cyanogenic moth *Z. filipendulae* indicates a connection

with cyanide detoxification [109]. β -Cyanoalanine synthase activity was higher in cyanide-tolerant than in cyanide-sensitive species [86]. Three studies analyzed a broad spectrum of insect species either by measuring rhodanese enzyme activities or by determining β -cyanoalanine as an indicator of β -cyanoalanine synthase activity [103, 106, 110]. However, in addition to the difficulty to compare enzyme activity and metabolite level *per se*, several other factors render a statement on the *in vivo* relevance of β -cyanoalanine synthase and rhodanese for cyanide detoxification in insects impossible: the low number of replicates [103], the use of specimen of different developmental stages and from different origins [103, 110], quantification by thin-layer chromatography, and long-term storage of specimen before analysis despite decomposition of metabolites over time [106, 110]. Thus, conclusions on the quantitative contribution of the two pathways of cyanide detoxification in insects cannot be drawn from these experiments. Nevertheless, the studies showed that both enzymes have a broad distribution among insects and many species possess both activities [30, 55, 103, 104, 110, 111]. β -Cyanoalanine synthase seems to be most relevant to protect arthropod herbivores feeding on cyanide-defended plants.

Most β -cyanoalanine synthase activity data for animals were generated with intact, partly dried and stored animal tissues, but some enzymes have been purified and characterized [84, 104]. In the nematode *Caenorhabditis elegans*, identification of the first animal members of the β -substituted alanine synthase family that do not belong to the cystathionine- β -synthases was achieved at the molecular level [85]. Biochemical characterization of the enzymes proposed roles in the regulation of the metabolism and the detoxification of cyanide, sulfide and S-sulfocysteine [84]. Although the enzymes possess *O*-acetylserine thiol lyase activity *in vitro*, an *in vivo* function in sulfur assimilation and cysteine biosynthesis is unlikely [84]. Further β -cyanoalanine synthases have been identified in the mite *Tetranychus urticae* and larvae of *P. rapae* [87, 95]. These first molecular data indicate an astonishing evolutionary background. In phylogenetic analyses, the nematode enzymes group together with plant *O*-acetylserine thiol lyases [84, 85] indicating a common origin of β -cyanoalanine synthases of both kingdoms, that is, acquisition of β -cyanoalanine synthases by gene duplication and partial neofunctionalization of a common ancestor. In contrast, the β -cyanoalanine synthases from the mite and the butterfly show amino acid sequence similarity with bacterial sequences [87, 95]. Phylogenetic analyses group the mite and butterfly enzymes among bacterial homologs from α - and β -proteobacteria, distant to other metazoan β -substituted alanine synthases [87, 95]. Genetic analyses such as determination of the GC-nucleotide content and mapping of adjacent genomic DNA have shown that these sequences belong to the arthropods' genome rather than to the genome of bacterial symbionts of the present species [87, 95]. As a likely explanation, the genes may have been acquired by horizontal gene transfer from bacteria, likely from symbiotic bacteria which lived in close association with ancestral arthropods [87]. Nevertheless, a common horizontal gene transfer event to arthropods seems to be unlikely as this would involve at least 13 independent gene losses each at a very specific point in the evolution [87]. Two independent gene transfer events from closely related bacterial donor species or a gene transfer from bacterium to mite followed by a second transfer event from an ancient mite to a Lepidopteran ancestor are discussed [87]. Interestingly, mites possess only one copy of the sequence in their genome, while some Lepidoptera seem to possess two or three copies [95], a phenomenon frequently observed for genes assimilated by horizontal gene transfer [112]. Horizontal gene transfer, in particular from bacteria and protists, but also

from plants and fungi, has contributed gravely to metazoan evolution. Its remnants besides the β -cyanoalanine synthases can be found in tens to hundreds of examples in nematodes, arthropods and chordates and are involved in main metabolic pathways and responses to environmental influences [113].

In general, proteins involved in the adaptation of herbivores to their host plants and, in particular, those catalyzing the detoxification and transport of host plant xenobiotics are thought to be under narrow transcriptional regulation [114]. For the mite β -cyanoalanine synthase, an induction by cyanide exposure over 30 generations led to a transcriptional response allowing for the identification of the detoxification enzyme [87]. Thus, these enzymes are among the most variable ones and play a key role in the adaptation to and population of new host plants [114]. As the relationship between herbivore and host plant is close and evolution favors adapted defense of the plant in order to diminish resource and tissue loss through predation [115], transcriptional responses could be discovered also in the host plant [114]. This close coevolution between herbivores and their host plants has therefore shaped both partners and is likely to underlie the higher cyanide tolerance of specialist herbivores on cyanogenic plants [30, 115].

6. Alternative herbivore strategies to cope with cyanogens

Next to efficient means of cyanide detoxification, herbivores have developed alternative ways to avoid intoxications when feeding on cyanide-defended plants. Often, the cyanide potential of food plants is below a toxic threshold [116]. As generalist herbivores usually change their food plants frequently, this allows them to mix a cyanide-rich diet with a diet low in cyanide to keep the overall cyanide intake below a toxic threshold [116]. Moreover, cyanogenic glucoside occurrence is often accompanied by a bitter taste of the potential food plant and many herbivores therefore avoid feeding on these plants if other host plants are available [116]. Nevertheless, in no-choice feeding experiments or if no other food plant is available in the habitat, herbivores may consume high amounts of cyanide-defended plants leading to intoxication or even death [116, 117]. Adaptations to reduce this risk include morphological, behavioral, physiological and biochemical mechanisms as outlined in the following paragraphs.

The mouthparts of herbivores from the Aphididae have evolved to specialized sucking styli which they insert through the apoplast into the sieve elements to suck phloem sap. This feeding mode avoids tissue disruption and therefore the mixing of plant cyanogenic glucosides and their spatially separated hydrolysis enzymes [41, 116, 118, 119]. In the lepidopteran specialist *Z. filipendulae* feeding on the cyanogenic glucoside-rich *L. corniculatus*, a leaf-snipping mode (leaving large portions of the plant tissue intact) in combination with a high feeding speed (shortening the time of a potential interaction between plant β -glucosidases and their substrates) decreases cyanogenic glucoside hydrolysis [120]. A similar leaf-snipping mode to avoid large-scale tissue damage was observed in a range of lepidopteran larvae not specialized on plants containing cyanogenic glucosides, including generalists as well as specialists on plants possessing another activated defense system, the glucosinolate-myrosinase-system [118]. All tested species tolerated high cyanogenic glucoside levels in their diet and excreted

intact cyanogenic glucosides with their frass [118]. This was mainly achieved by avoiding cyanogenic glucoside hydrolysis through the feeding mode [118] indicating that leaf-snipping might be an ancient trait which evolved and has been maintained in lepidopteran herbivores in response to the frequent occurrence of activated plant defenses.

As a physiological adaptation, the strongly alkaline midgut pH found in some generalist and specialist herbivores allows for the inhibition of the ingested plant β -glucosidases and avoidance of cyanide liberation [118, 120] in contrast to other species with a slightly acidic midgut pH that are prone to cyanide intoxication [121, 122]. Further, properties and expression of the herbivore's endogenous β -glucosidases have undergone adaptational adjustments to reduce cyanide release from ingested plant material. As an example, the β -glucosidases localized in the saliva and midgut lumen of the cyanogenic glucoside-feeding specialist *Z. filipendulae* have lost their activity toward their host plants' cyanogenic glucosides, linamarin and lotaustralin [120]. Larvae of *Diatraea saccharalis* (Lepidoptera:Crambidae) are adapted to a cyanogenic diet by reducing the expression of aryl β -glucosidase in their midgut, thus decreasing cyanogenic glucoside catabolism and cyanide liberation [123]. A unique alternative strategy to avoid cyanide release which relies on metabolism of the cyanogenic glucoside before hydrolysis has only been described for larvae of *Heliconius sara* (Lepidoptera:Nymphalidae) so far [124]. Larvae of *H. sara* feed exclusively on leaves of *Passiflora auriculata* (Passifloraceae) with cyanocyclopentenyl glucosides as major cyanogens. Upon ingestion of leaf material, the nitrile group of the main cyanogenic glucoside is specifically replaced by a thiol to produce a compound which is not a cyanide precursor anymore [124].

Yet another mechanism protects millipede species (Diplopoda) from cyanide poisoning. These animals possess a highly tolerant cytochrome c oxidase, making cyanide poisoning less effective [125]. Instead of or in combination with a cyanide-resistant terminal oxidase, a complete cyanide-insensitive oxidative pathway has also been proposed [126]. Studies on the respiratory rate of larvae of the lepidopteran generalist herbivore, *Spodoptera eridania* (Lepidoptera:Noctuidae), also showed high cyanide tolerance, possibly indicating insensitivity of their terminal oxidase [126]. Other mechanisms of cyanide tolerance may await their discovery. In addition, a possible contribution of enzyme activities from closely associated anaerobic cyanide-resistant gut microbes has to be clarified [127].

7. Cyanogenic compounds in arthropod herbivores

7.1. Occurrence of cyanogenic compounds in arthropods

Arthropods are the only phylum of animals in which biosynthesis or sequestration of cyanogenic compounds has been shown [15]. Within arthropods, the presence of cyanogenic glucosides appears to be restricted to millipedes (Diplopoda), centipedes (Chilopoda) and three orders within the Insecta (Lepidoptera, Coleoptera and Hemiptera) [128]. The Lepidoptera and Hemiptera are the only groups containing cyanogenic compounds with aliphatic side chains, while in the others groups of arthropods, cyanogenic compounds possess aromatic side chains [4, 129]. Among the most intensely studied species, larvae of *Z. filipendulae* spe-

cialized on cyanogenic plants are able to *de novo* biosynthesize cyanogenic glucosides, but can also sequester cyanogenic glucosides from their food plants [4, 17, 130] (see Sections 7.2 and 7.3). Both *de novo* biosynthesis and sequestration of cyanogenic glucosides have also been reported for larvae of *Euptoieta hegesia* (Lepidoptera:Nymphalidae) based on the detection of cyanide after larval feeding on acyanogenic plants and an increase of cyanide formation upon transfer to cyanogenic glucoside-defended plants [131]. Species of the genus *Heliconius* are also able to synthesize and to sequester cyanogenic glucosides based on the pattern of compounds detected in the insects relative to those found in the food plants [124, 132]. In contrast to lepidopterans, millipedes do not store glycosides, but unglycosylated cyanogens, and do not sequester cyanide precursors from their diet as discussed in Ref. [133].

7.2. *De novo* biosynthesis of cyanogenic compounds in arthropods

First indications for *de novo* biosynthesis of cyanogenic glucosides in a herbivore came from experiments conducted by Jones and coworkers in 1962. They showed that larvae of *Z. filipendulae* release cyanide upon tissue disruption also when raised on food plants devoid of cyanogenic glucosides [109]. Feeding experiments with ¹³C-labeled valine and isoleucine showed the incorporation of the isotope label into linamarin and lotaustralin and thereby provided evidence for *de novo* biosynthesis of cyanogenic glucosides in *Z. filipendulae* and the butterfly *Heliconius melpomene* (Lepidoptera:Nymphalidae) [132, 134]. The complete biosynthetic pathway of cyanogenic glucosides in *Z. filipendulae* was elucidated in 2011 including pathway intermediates and involved enzymes [16, 17, 19, 134, 135]. This revealed high similarity to the biosynthesis of cyanogenic glucosides in plants with two cytochrome P450 enzymes catalyzing the conversion of the precursor amino acid to a cyanohydrin via an aldoxime and a glucosyltransferase responsible for the final glycosylation step (see **Figure 4**). Several butterfly species of the Papilionoidea also contain linamarin and, in part, lotaustralin in different life stages [128, 132, 136, 137] due to *de novo* biosynthesis rather than sequestration as the larval host plants, in most cases, do not form aliphatic cyanogenic glucosides [4].

Among arthropods, millipedes also contain cyanogenic compounds, namely cyanohydrins such as mandelonitrile, and use them as defense against predators as discussed by Shear [133]. Synthesis of cyanide and cyanohydrins such as mandelonitrile was demonstrated for different species of millipedes using feeding tests with ¹⁴C-phenylalanine and further radioactively labeled precursors [14, 107, 138]. As this resulted in labeling of phenylacetaldoxime and phenylacetoneitrile as potential pathway intermediates, a biosynthesis pathway very alike the one described in plants and later in insects was proposed [14, 107, 138] (**Figure 4**). The unwanted release of cyanide is prevented by specifically shaped, two chamber glands where cyanide precursor and the hydrolyzing enzyme α -hydroxynitrile lyase are stored separately [107, 133]. In the α -hydroxynitrile chamber, organic acids generate low pH values to stabilize the α -hydroxynitrile [133]. Upon attack by a predator, gland secretions are mixed to generate cyanide. This mechanism allows the millipede to liberate cyanide in a controlled way, thereby economizing its chemical defense and protecting its own tissue from poisoning.

Although the presence of cyanogenic glucosides and, in part, their *de novo* biosynthesis has been described in selected species of the centipedes [139], millipedes and insects, and these

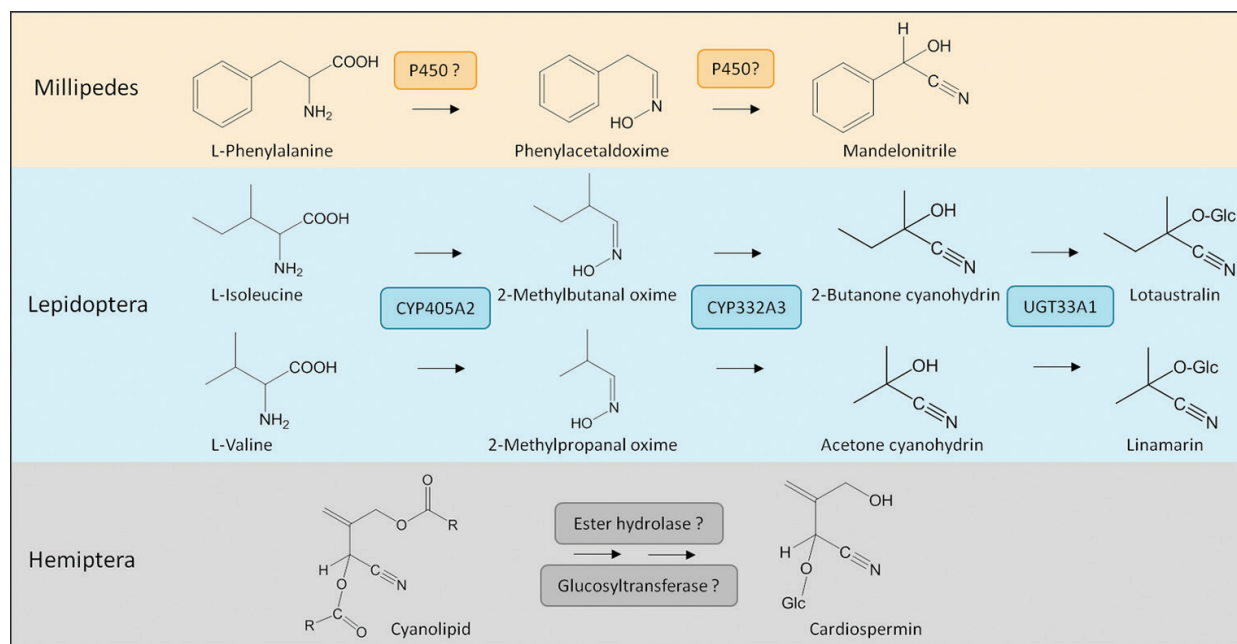


Figure 4. Biosynthesis of cyanogenic defense compounds in arthropods. Shown are the most widespread compounds and the enzymes characterized or proposed for the corresponding pathway. The biosynthesis of linamarin and lotaustralin in *Zygaena filipendulae* has been completely elucidated [16]. For millipedes, a similar reaction pathway is indicated by the reaction intermediates identified so far [14, 107, 138]. Cardiospermin is found in species of the Hemiptera, but its biosynthesis pathway is still unknown. Cyanolipids have been proposed as precursors [129].

phyla derive from a common ancestor, the absence of cyanogens in most insect species and the diversity of cyanogenic glucosides in combination with the long time span since their diversion (>390 MYA) [140] indicate that the biosynthetic pathways evolved independently in the different phyla [4]. Interestingly, insects biosynthesizing cyanogenic glucosides *de novo* also express both enzymes needed for their degradation and cyanide liberation, β -glucosidase and α -hydroxynitrile lyase [141].

7.3. Sequestration of cyanogenic compounds from the food plants

Most data known on sequestration of cyanogenic compounds in insects were generated using larvae of *Z. filipendulae* as a model. *Z. filipendulae* sequesters the cyanogenic glucosides linamarin and lotaustralin from its host plant *L. corniculatus* as intact glucosides, transports them into all tissues and retains them upon metamorphosis where they can be found in all tissues, too, as studies with isotopically labelled linamarin and lotaustralin have shown [142]. Interestingly, larvae of *Z. filipendulae* are also able to sequester the aromatic cyanogenic glucoside prunasin which they do not come in contact with naturally [118]. This indicates a versatile transport mechanism [118] and an evolutionary origin from a more general mechanism to deal with host plant xenobiotics.

Sequestration of cyanogens has also been shown for gynocardin, a cyclic α -hydroxynitrile glucoside, in *Acraea horta* (Lepidoptera:Nymphalidae) [143] and for sarmentosin, a β -hydroxynitrile, in *Parnassius phoebus* (Lepidoptera:Papilionidae) [144]. Although the latter compound is not cyanogenic *per se*, enzymatic catalysis may lead to the liberation of

cyanide. Alternatively, a function of the intact compound as deterrent against predators is discussed [144].

Larvae of the bug *Leptocoris isolata* (Hemiptera:Serinethinae), but not the adult bugs, were shown to contain cyanogenic glucosides such as cardiospermin not present in their host plant [129]. However, their host plant produces cyanolipids containing the same aglucone. Therefore, it was proposed that this species recruited enzymes of xenobiotic metabolism to transform sequestered cyanolipids into cyanogenic glucosides for use in its own defense [129] (Figure 4).

7.4. Benefit of cyanogenesis for herbivores

Cyanogenic glucosides derived from *de novo* biosynthesis and sequestration may play a pivotal role in herbivore defense against predators as has been demonstrated with the model species *Z. filipendulae*. Larvae of *Z. filipendulae* react to aggression such as pricking by release of a defensive fluid from their dorsal cavities. This fluid contains, next to the cyanide detoxification product β -cyanoalanine, high amounts of the two aliphatic cyanogenic glucosides, linamarin and lotaustralin, and, among other proteins, their hydrolysis enzyme, β -glucosidase [100, 145]. Thus, cyanogenic glucosides and cyanide are used for deterrence and intoxication of potential predators. If whole larvae are ingested by a mammalian predator, another mechanism comes into effect. The larval hemolymph contains high amounts of β -glucosidase inactivated by a high pH value. Upon release of these proteins into the highly acidic environment of the predator's stomach, the β -glucosidase gets activated and cyanogenic glucoside hydrolysis leads to cyanide poisoning of the predator (discussed in Ref. [4]).

Nevertheless, cyanide alone is not always efficient for the animal's defense. It has been shown that in millipedes, not cyanide itself but the second product of mandelonitrile hydrolysis, benzaldehyde, is repellent to ants [49]. In contrast, for the intact cyanogenic glucoside cardiospermin a deterrent effect on ants has been shown which could not be observed for any cyanogenic glucoside before [129]. Thus, predating insects facing cyanide in their prey may have evolved a sensitive perception of substances with stronger odor or taste usually occurring alongside cyanide [49]. Alternatively, it was proposed that cyanide is the main means of defense against vertebrate predators, while benzaldehyde is used to repel arthropod enemies [133]. Phylogenetic and physiological data indicate that cyanogenesis as defense strategy has been lost and replaced by ancient phenolic defense compounds in some groups among the Polydesmida, mainly those unlikely to be targeted by vertebrate predators [133].

Next to their defensive roles, linamarin and lotaustralin are also used as nitrogen sources for chitin biosynthesis based on their turnover during metamorphosis [146]. During the formation of the pupal cuticle, cyanogenic glucosides are a key nitrogen source [146, 147]. However, mobilization through β -cyanoalanine synthase and nitrilase/nitrile hydratase leading to the formation of asparagine and aspartate similar to plants [148] has not been demonstrated in insects so far. The efficient transport of cyanogenic glucosides in *Z. filipendulae* is nevertheless evident based on the occurrence of these compounds in the wings, an organ devoid of any biosynthetic activity [146]. The important role of sequestered and *de novo*-biosynthesized cyanogenic glucosides for the defense and metabolism of the lepidopteran specialist *Z. filipendu-*

lae was additionally proven by the finding that male adult butterflies transfer the compounds to their partners as nuptial gift upon mating [142, 147].

Based on transcriptional and metabolite analyses [149], it has been hypothesized that the biosynthesis of cyanogenic glucosides in arthropods is older than their sequestration [149]. The biosynthesis is thought to have been constitutive in the ancestors of *Zygaena* which did not live on cyanogenic plants (but on Celastraceae) and were, therefore, not able to receive cyanogenic glucosides from their host plants. Upon exploration of cyanogenic plants, the insects' endogenous biosynthesis became inducible as sequestration helped to reduce metabolic costs for *de novo* biosynthesis [149]. Thus, the ability to handle and *de novo* biosynthesize cyanogenic glucosides allowed the moths to extend their host plant range and to even exploit the newly acquired host plants, cyanogenic glucoside-producing Fabaceae, to conserve energy and nutrients otherwise needed for the biosynthesis of these compounds [4, 128].

8. Conclusions and perspectives

The past 15 years have witnessed an enormous progress in our understanding of herbivore adaptational mechanisms to plant cyanide defenses and their evolution. A lot of the present knowledge has been acquired through the application of state-of-the-art analytical and molecular tools as well as imaging techniques to the model species *Z. filipendulae*, a well-known specialist on cyanide-defended plants which is able to sequester and *de novo* synthesize cyanogenic glucosides. Detailed studies of its feeding mode and gut physiology, the properties of its gut β -glucosidase and the evolution of its own biosynthetic pathway for cyanogenic glucosides have illuminated the role of cyanogenic glucosides as defenses and nitrogen storage compounds which have shaped coevolutionary relations between herbivores and their host plants. The availability of genome and transcriptome data of diverse animal species has fueled studies into cyanide detoxification mechanisms in herbivores. Together with modern analytical techniques, molecular biology has enabled identification of the first animal β -cyanoalanine synthases. As an interesting evolutionary background, these studies revealed that arthropod β -cyanoalanine synthases have likely been acquired through horizontal gene transfer from microbial gut symbionts. Proof of rhodanese activity of putative enzymes from arthropod databases still needs to be provided.

Future research will have to extend the present insights by studying a broader range of species with respect to their behavioral, physiological and biochemical adaptations to cyanogens. Besides the identification and detailed characterization of cyanide detoxification enzymes from additional species, transporters involved in cyanogen sequestration will be an interesting target of future investigations. In addition, experimental proof of essential roles of herbivore proteins involved in overcoming plant cyanide defenses might become possible *in vivo* through RNA interference or genome editing. Taken together, studies on herbivore adaptations to plant cyanide defenses are a prime example of the added value of multidisciplinary research combining ecology, physiology, biochemistry, systematics and genetics to provide insights into coevolution of herbivores and their food plants.

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