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Review

Cyanogenic glucosides and plant–insect interactions

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Abstract

Cyanogenic glucosides are phytoanticipins known to be present in more than 2500 plant species. They are considered to have an important role in plant defense against herbivores due to bitter taste and release of toxic hydrogen cyanide upon tissue disruption. Some specialized herbivores, especially insects, preferentially feed on cyanogenic plants. Such herbivores have acquired the ability to metabolize cyanogenic glucosides or to sequester them for use in their predator defense. A few species of Arthropoda (within Diplopoda, Chilopoda, Insecta) are able to de novo synthesize cyanogenic glucosides and, in addition, some of these species are able to sequester cyanogenic glucosides from their host plant (Zygaenidae). Evolutionary aspects of these unique plant–insect interactions with focus on the enzyme systems involved in synthesis and degradation of cyanogenic glucosides are discussed.
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1. Introduction

Cyanogenic glucosides (CNGs) are phytoanticipins widely distributed in the plant kingdom (Conn, 1980; Møller and Seigler, 1999; Poulton, 1990). They are present in more than 2500 different plant species including ferns, gymnosperms and angiosperms. This indicates that the ability of plants to produce CNGs is ancient. In addition, CNGs have been found in a few arthropod clades. CNGs are β-glucosides of α-hydroxynitriles derived from the aliphatic protein amino acids L-valine, L-isoleucine and L-leucine, from the aromatic amino acids L-phenylalanine and L-tyrosine and from the aliphatic non-protein amino acid cyclopentenyl-glycine (Fig. 1). In plants, CNGs are stored in the vacuoles (Vetter, 2000). When plant tissue is disrupted e.g. by herbivore attack, CNGs are brought into contact with β-glucosidases and α-hydroxynitrile lyases that hydrolyze the CNGs and thereby cause release of toxic hydrogen cyanide (HCN) (Fig. 1). This binary system—two sets of components that, when separated, are chemically inert—provides plants with an immediate defense against intruding herbivores and pathogens that cause tissue damage.

Cyanide is a toxic substance, mainly due to its affinity for the terminal cytochrome oxidase in the mitochondrial respiratory pathway (Brattsten et al., 1983). The lethal dose of cyanide for vertebrates lies in the range of 35–150 μmol kg^{-1}, if applied in a single dose. Much higher amounts of HCN can be tolerated if consumed or administered over a longer period (Davis and Nahrstedt, 1985). Biosynthesis and degradation of CNGs are well documented in many plants (Jones et al., 2000; Lechtenberg and Nahrstedt, 1999).

For most plants it has been hypothesized that CNGs are involved in plant defense against herbivores due to release of toxic HCN (Nahrstedt, 1996). CNGs are, however, also known to act as both feeding deterrents and phagostimulants for herbivores that are specialists on plants containing CNGs (reviewed in Gleadow and
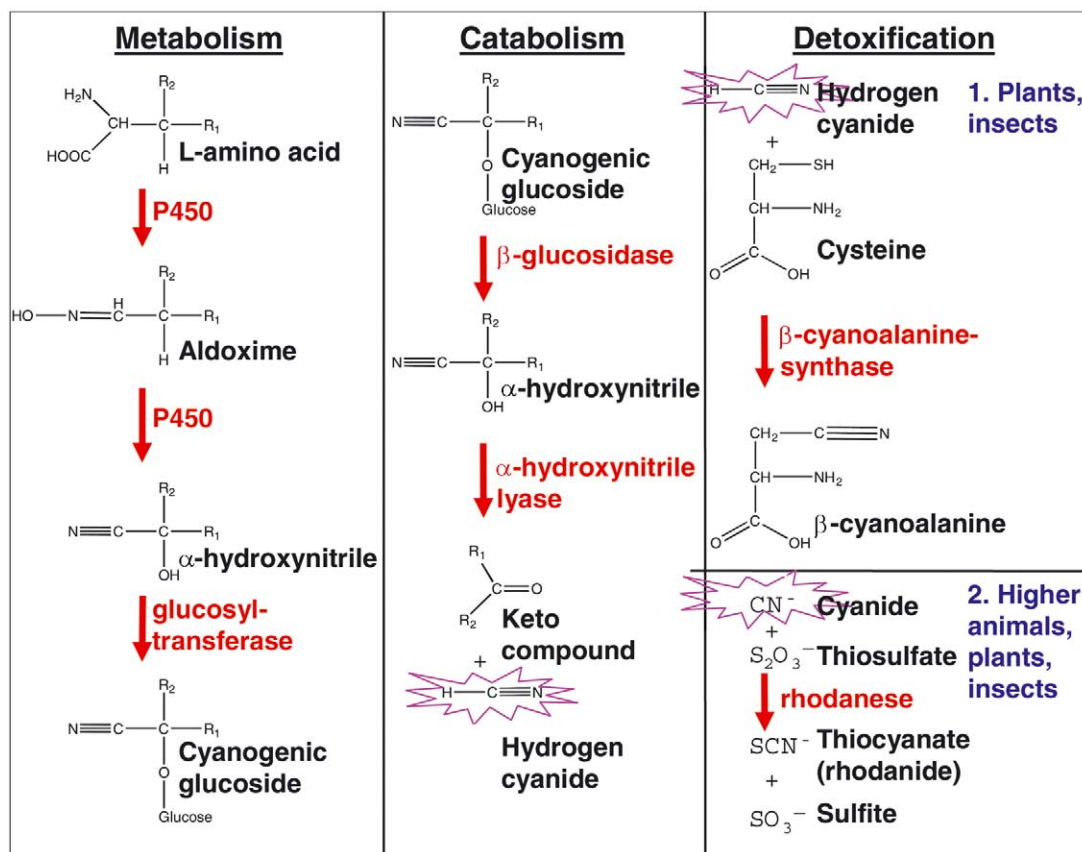

Fig. 1. Biosynthesis, catabolism and detoxification of CNGs in plants, insects and higher animals. Enzymes involved are shown in red. HCN is highlighted in purple.

Woodrow, 2002). This review will aim to summarize current knowledge on CNGs and their synthesis and degradation in insects compared to plants, and to discuss possible evolutionary implications of these relationships.

2. Biosynthesis, degradation and detoxification of cyanogenic glucosides

The main metabolic processes resulting in synthesis, degradation and detoxification of CNGs in plants are shown in Fig. 1. The first two committed steps in CNG biosynthesis are catalyzed by cytochromes P450 (Fig. 1). The first P450 catalyzed step proceeds via two successive N-hydroxylations of the amino group of the parent amino acid, followed by decarboxylation and dehydration (Sibbesen et al., 1994). The aldoxime formed is subsequently converted to an α-hydroxynitrile through the action of a second cytochrome P450 (Bak et al., 1998; Kahn et al., 1997) (Fig. 1). This reaction involves an initial dehydration reaction that forms a nitrile and is followed by hydroxylation of the alpha carbon to generate a cyanohydrin. The final step in CNG synthesis, glycosylation of the cyanohydrin moiety, is catalyzed by a UDPG-glycosyltransferase (Jones et al., 1999) (Fig. 1). Catabolism of CNGs is initiated by enzymatic hydrolysis by a β-glucosidase to afford the corresponding α-hydroxynitrile, which at pH values above 6 spontaneously dissociates into a sugar, a keto compound, and HCN (Fig. 1). At lower pH values, the dissociation reaction is catalyzed by an α-hydroxynitrile lyase. HCN is detoxified by two main reactions (Møller and Poulton, 1993) (Fig. 1). The first route involves the formation of β-cyanoalanine from cysteine and is catalyzed by β-cyanoalanine-synthase (Fig. 1, route 1). β-Cyanoalanine is subsequently converted into asparagine (Miller and Conn, 1980). The second route proceeds by conversion of HCN into thiocyanate and is catalyzed by rhodanese (Bordo and Bork, 2002) (Fig. 1, route 2). The detoxification route involving β-cyanoalanine is common in plants and possibly also in insects, while the thiocyanate pathway occurs mainly in vertebrates but also in some plants and insects.

Cytochromes P450 are heme-thiolate monooxygenases ubiquitously found in all kingdoms. They constitute a large group of polyphyletic proteins with amino acid sequence similarity as low as 20% within species. In eukaryotes, cytochromes P450 are type II membrane enzymes, N-terminally anchored to the endoplasmatic membrane. In prokaryotes, cytochromes P450 are soluble (Werck-Reichhart and Feyereisen, 2000). In insects and plants, cytochromes P450 are encoded by some of the largest multigene families, with 89 genes in the Drosophila melanogaster (Endopterygota) genome (Tijet et al., 2001) (http://p450.antibes.-inra.fr/) (Fig. 2), 100 genes in the Anopheles gambiae
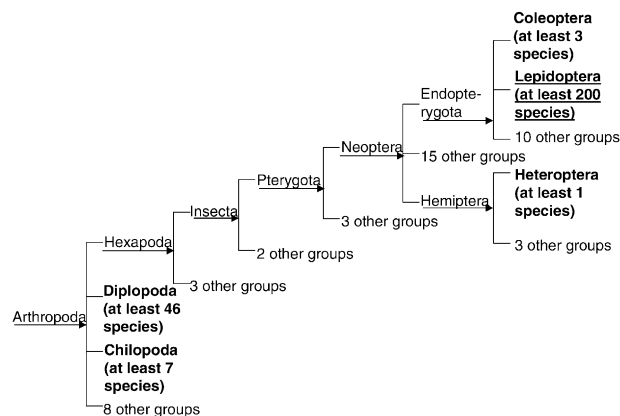

Fig. 2. Simplified schematic evolutionary tree for Arthropoda. Arthropod groups encompassing species that contain aromatic CNGs are shown in bold whereas the group that contains aliphatic CNGs is shown in bold and underlined. The number of species that contain CNGs within each of the groups is listed in parentheses.

(Endopterygota) genome (Ranson et al., 2002) (http://p450.antibes.inra.fr/) (Fig. 2) and 272 genes in the Arabidopsis thaliana genome (Werck-Reichhart et al., 2000) (http://biobase.dk/P450/). Cytochromes P450 catalyze a highly diverse range of chemical reactions that include C-hydroxylations and epoxidations, N- and S-oxidations, dehydrations and O-, N- and S-dealkylations (Feyereisen, 1999; Halkier, 1996; Morant et al., 2003).

In insects, cytochromes P450 are involved in biosynthesis of ecdysteroids and juvenile hormones as well as in metabolism and detoxification of insecticides. Cytochromes P450 play crucial roles in defense against natural products that insects have to fend off in order to be able to feed on otherwise toxic plants. The ability of an insect cytochrome P450 to metabolize a specific natural product is often the key to the adaptation of insect herbivores to their host plants (Feyereisen, 1999). In plants, cytochromes P450 are involved in biosynthesis of a vast array of natural products involved in plant defense as well as many other biosynthetic pathways (for recent reviews see Morant et al., 2003; Werck-Reichhart et al., 2002).

The final step in biosynthesis of CNGs is catalyzed by a Family 1 glycosyltransferase (Jones et al., 1999; Vogt and Jones, 2000). Family 1 glycosyltransferases are soluble proteins with a molecular mass of 45–60 kDa, which utilize UDP-activated sugar moieties as the donor molecules to glycosylate the acceptor molecules. Glycosyltransferases generally exhibit a low degree of overall sequence similarity, and are often regioselective or regiospecific rather than highly substrate specific (Hansen et al., 2003). Typically, the first committed step in a biosynthetic pathway is catalyzed by an enzyme with high substrate specificity. This serves to limit the number of available substrates for subsequent enzymes in the same pathway. These enzymes may thus possess a wider substrate specificity that provides overall

metabolic flexibility, yet desired specificity with a limited number of genes (Vogt and Jones, 2000). As for cytochromes P450, Family 1 glycosyltransferases are encoded by a multigene family and are ubiquitously found in plants, animals, fungi, bacteria and viruses (Paquette et al., 2003).

In plants, degradation of CNGs is catalyzed by β-glucosidases and α-hydroxynitrile lyases (Conn, 1980; Hösel and Conn, 1982; Poulton, 1990). β-Glucosidases that catalyze the hydrolysis of glycosidic linkage in aryl and alkyl β-glucosides and in cellubiose are present in bacteria, fungi, plants and animals. In contrast to the well characterized β-glucosidases involved in CNG catabolism in plants (Cicek et al., 2000; Cicek and Esen, 1998; Czjzek et al., 2000), only little is known about these insect β-glucosidases and their substrate specificity. β-Glucosidases generally have a subunit molecular mass of 55–65 kDa, acidic pH optima (pH 5-6) and an absolute specificity towards β-glucosides (Esen, 1993). Plant β-glucosidases involved in cleavage of CNGs exhibit a high specificity towards the aglycone moiety of CNGs present in the same plant species (Hösel et al., 1987; Hösel and Conn, 1982; Nahrstedt, 1985).

α-Hydroxynitrile lyases have been characterized in plants (Hu and Poulton, 1997, 1999; Wajant and Pfizenmaier, 1996) and in a single case from an insect (Müller and Nahrstedt, 1990). In plants, they appear to be located in the same tissues as the CNG degrading β-glucosidases, though their activity is observed in protein bodies (Swain et al., 1992), instead of in chloroplasts or apoplastic space as typically reported for β-glucosidases (Hickel et al., 1996). α-Hydroxynitrile lyases constitute two broad phylogenetically distinct groups that have convergently evolved to the same function (Møller and Poulton, 1993). One homogeneous group comprises monomeric FAD-containing glycosylated enzymes. These enzymes have only been found in two subfamilies within the Rosaceae and utilize the aromatic cyanohydrin mandelonitrile as a substrate. The other group is more heterogeneous and comprises dimeric or oligomeric non-FAD containing enzymes that typically are not glycosylated. These enzymes have been found in di- and monocotyledonous plant families, and their natural substrates may be *p*-hydroxymandelonitrile as well as acetone cyanohydrin, the latter being the most common substrate. This group of non-FAD containing α-hydroxynitrile lyases may be divided into subgroups depending on highest sequence similarity to serine carboxypeptidases, alcohol dehydrogenases or to rice proteins of unknown function (Hickel et al., 1996; Trummler and Wajant, 1997).

β-Cyanoalanine synthase activity is generally found in plants (Miller and Conn, 1980) and plays a pivotal role in detoxification of HCN released as a result of cleavage of cyanogenic glucosides, or formed in stochiometric amounts with ethylene by the enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (Yip and Yang, 1988). β-Cyanoalanine synthase activity in plants and insects is primarily located in mitochondria, the organelle that is most vulnerable to HCN toxification (Meyers and Ahmad, 1991). In plants, β-cyanoalanine synthase has pyridoxal phosphate as a cofactor (Ikegami et al., 1988) and is a member of an ancestral family of β-substituted alanine synthases that also includes cysteine synthase (Ikegami and Murakoshi, 1994). Cysteine synthase also possesses β-cyanoalanine synthase activity and vice versa. Typically, β-cyanoalanine is converted into asparagine by the action of β-cyanoalanine hydrolase (Catric et al., 1972). β-Cyanoalanine is a potent neurotoxin and its accumulation in some plants may serve to deter predators (Ressler et al., 1969).

In contrast to β-cyanoalanine synthase, rhodanese is not ubiquitously present in plants. The in vivo function of rhodanese is not well understood. In those species of higher animals, plants and insects in which rhodanese is present, it is thought to play a role in cyanide detoxification (Beesley et al., 1985). In plants, this assignment is supported by high levels of rhodanese activity in 3-day-old etiolated *Sorghum bicolor* seedlings (Miller and Conn, 1980). In these seedlings, the cyanide potential is exceptionally high (Halkier and Møller, 1989). Rhodanese enzymes probably serve a variety of other functions, the most important of which is to donate sulfur to proteins (Bordo and Bork, 2002).

3. Cyanogenic glucosides and plant–herbivore interactions

Herbivores react very differently to the presence of CNGs in their diet. Nearly all of the variability in the effectiveness of CNGs in plant defense against herbivory is explained by four confounding factors (Gleadow and Woodrow, 2002). First, the concentration of CNGs in a host plant may be below threshold toxicity. Second, the herbivore may be a specialist that has evolved mechanisms to cope with high levels of HCN in the diet. Third, the cyanogenic plant may be consumed as part of a mixed diet and the toxicity of CNGs in this way diluted to below threshold value. Fourth, the mode of herbivore feeding may be adapted to minimize tissue damage to leaves (e.g. aphids, which are phloem feeders) to limit exposure of CNGs to degradative β-glucosidases (Gleadow and Woodrow, 2002). It appears that the prime deterrent effect of CNGs is linked to the keto compound released in equimolar amounts to HCN during CNG degradation, rather than to the CNG or HCN (Jones, 1988). The biosynthetic pathway for CNGs is highly channeled, preventing intermediates to dissociate from the enzyme complex (Møller and Conn, 1980; Tattersall et al., in press). Accordingly, the biosynthetic intermediates are not likely to act as deterrents to herbivores. In contrast, degradation of CNGs may result in accumulation of

cyanohydrins, keto compounds or aldehydes, HCN, β-cyanoalanine, thiocyanate and sulfite (Fig. 1). Each of these compounds may be envisioned to possess defensive properties: CNGs have a bitter taste and have been shown to act as feeding deterrents; aldehydes and ketones posses cytotoxic activities; HCN is a powerful inhibitor of respiration and of enzymes that contain heavy metals; β-cyanoalanine is a neurotoxin (Nahrstedt, 1985; Ressler et al., 1969) and thiocyanate and sulfite are enzyme inhibitors.

A range of plant natural products comprising alkaloids, phenylpropanoids, terpenoids, glucosinolates and CNGs have been tested for their deterrence and post-ingestional effects on *Schistocerca americana* (grasshopper, Neoptera) and *Hypera brunneipennis* (alfalfa weevil, Coleoptera) (Fig. 2). None of the compounds tested were detrimental to the grasshoppers, but eight out of ten compounds deterred feeding (Bernays, 1991; Bernays and Cornelius, 1992). Weevils were deterred by 11 of 15 compounds but again, none of these had detrimental effects. Therefore, grasshoppers and weevils seem to be behaviorally more sensitive to plant natural products, including CNGs, than required for reasonable protection from ingestion (Bernays, 1991). This may also apply to other insects that are deterred by CNGs.

The entire pathway for synthesis of the aromatic tyrosine-derived CNG dhurrin in *S. bicolor* has been transferred to *A. thaliana* using gene technology to insert the three *S. bicolor* genes *CYP79A1*, *CYP71E1* and *UGT85B1* (Tattersall et al., 2001). The accumulation of dhurrin in the transgenic *A. thaliana* plants prevented feeding by *Phyllotreta nemorum* (Coleoptera) (Fig. 2), thus unambiguously demonstrating that CNGs can confer resistance to herbivory (Tattersall et al., 2001).

In nature, some *Lotus corniculatus* plants are cyanogenic due to the presence of the two cyanogenic glucosides linamarin and lotaustralin, whereas other plants are acyanogenic either because they do not synthesize CNGs, or because they lack the β-glucosidase required for degradation and HCN release (Jones, 1962, 1988). Between different insects, response to the presence of CNGs in *L. corniculatus* leaves varies from total indifference to evident distaste. After starvation, insects are generally more willing to feed on cyanogenic *L. corniculatus* leaves. This indicates that for each herbivore, the deterrent capabilities of CNGs is dependent on the immediate demand for food calories (Compton and Jones, 1985). Among those species that rely on *L. corniculatus* as a major food source, there was a general lack of selectivity against CNGs, which probably reflects specialized adaptations for a cyanogenic diet. High tolerance to CNGs may be characteristic of many polyphagous Lepidoptera species, and accordingly, the role of CNGs in protection of plants from herbivores must be assessed on a species to species basis.

As the above examples imply, the primary defensive role of CNGs in plants may be as a feeding deterrent and not as a toxin (Compton and Jones, 1985). In some plants, CNGs may serve as a warning to generalist herbivores that the plant is unpalatable. CNGs are well suited to such a role, because they are recognized by a wide variety of herbivores, are a relatively cheap type of plant defense, and because HCN liberation only occurs after tissue damage, hence conserving materials and reducing the risks that adapted herbivores gain the ability to use HCN as an attractant (Compton and Jones, 1985). In conclusion, the most likely overall function of CNGs appears to be to deter herbivores that would casually try to feed on cyanogenic plants (Jones, 1988).

4. Cyanogenic glucosides in Arthropoda

In contrast to the taxonomically widespread distribution of CNGs within the plant kingdom, presence of CNGs in animals appears to be restricted to a single phylum out of the currently known 31, namely the Arthropoda (Duffey, 1981; Nahrstedt, 1996) (Fig. 2). Within arthropods, presence of CNGs seems to be restricted to members of Chilopoda (centipedes), Diplopoda (millipedes) and in particular to Insecta (Davis and Nahrstedt, 1985). Within Insecta, CNGs have hitherto only been found in Coleoptera (beetles), Heteroptera (true bugs) and in particular in the Lepidoptera (butterflies and moths) (Nahrstedt, 1988) (Fig. 2).

Chilopoda, Diplopoda and some Coleoptera (*Paropsis atomaria, Chrysophtharta variicollis* and *C. amoena*) contain aromatic CNGs in their defensive secretions. Three beetle species appear to *de novo* synthesize CNGs as these are not present in their diet (Davis and Nahrstedt, 1985). Two species of diplopods (*Oxidus gracilis* and *Harpaphe haydeniana*) have evolved biochemical pathways for cyanogenic glucoside biosynthesis and degradation that involve very similar or identical intermediates compared to those known to be used by higher plants (Duffey, 1981). *H. haydeniana* has cyanogenic glands that contain β-glucosidase and α-hydroxynitrile lyase activity, physically separated from the part containing CNGs. This prevent untimely release of HCN (Duffey and Towers, 1978), but offers the possibility of immediate and combined ejection and thereby mimics the phytoanticipin defense effect in plants.

In contrast to other arthropod groups, many members of the Lepidoptera are able to de novo synthesize CNGs as well as to sequester CNGs from their host plants (see Sections 5 and 6). Only a single species of another arthropod group, the Heteroptera, has been proposed to be able to sequester CNGs from its host plant (Braekman et al., 1982). Furthermore, members of the Lepidoptera contain mainly aliphatic CNGs as opposed to other arthropod groups that are only known to contain aromatic CNGs.

Insecta evolved at least 390 Myr ago (Gaunt and Miles, 2002), so Diplopoda and Chilopoda have evolved from a common ancestor they shared with Hexapoda at an earlier time point. This time span combined with the fact that many hexapod groups do not contain CNGs, and that those groups that do, contain different types of CNGs, points to convergent evolution of CNGs in these clades rather than to homology (Duffey, 1981). It should be emphasized that to date, the presence of CNGs has only been examined in a few species of arthropods, so the distribution of CNGs may in fact be broader than currently recognized.

5. Cyanogenic glucosides in Zygaenidae (foresters and burnets)

Resistance of *Zygaena* species to HCN has been well known from the beginning of the 20th century (Davis and Nahrstedt, 1985; Levinson et al., 1973). *Zygaena* species can for example remain in a concentrated atmosphere of HCN for an hour and still revive quickly when removed to clean air (Naumann et al., 1999). In 1962, the release of HCN from crushed tissues of several *Zygaena* species was shown even after the insects were reared on acyanogenic plants. This observation suggested that *Zygaena* was able to de novo synthesize CNGs (Davis and Nahrstedt, 1982; 1987; Franzl et al., 1986; Holzkamp and Nahrstedt, 1994; Jones et al., 1962). With the identification of β-cyanoalanine synthase in *Zygaena* larvae (Witthohn and Naumann, 1987a), it became apparent that *Zygaena* species possess the complete enzyme systems to effectively produce, control and detoxify HCN. This defense system is proposed to have evolved as a result of a strong selection pressure for protection against insectivores (Nahrstedt, 1993). The potential of Zygaenidae species to detoxify HCN as well as to de novo synthesize CNGs, is probably a basic characteristic of Zygaenidae, and may have enabled some species to commence feeding on cyanogenic plants. This would be in agreement with the observed shift of host-plant specificity from Celastraceae to Fabaceae within the Zygaenidae. This shift seems to have been very successful as evidenced by the subsequent radiation of the subgenera *Zygaena* and *Argumentia* (Zygaenidae) (Müller et al., 1993). As a special adaptation, some highly evolved *Zygaena* species, such as *Z. trifolii*, have acquired the ability to sequester CNGs from their host plants. Accordingly, these moths manage to optimize their supply of CNGs by feeding on appropriate plant sources while minimizing the energy spent to achieve this goal (Nahrstedt, 1988).

5.1. Linamarin and lotaustralin distribution in Zygaenidae

The Zygaenidae are currently divided into four subfamilies of which CNGs have been found in three:

Zygaeninae, Procridinae and Chalcosiinae (Fig. 3). In addition, CNGs have been found in Charideinae and Anomoeotinae, two groups that were formerly placed in the Zygaenidae, but whose positions are currently unresolved. A total of 45 species from these five groups have been shown to contain the CNGs linamarin and lotaustralin (Fig. 4) independently of the presence or absence of CNGs in the food plants provided (Davis and Nahrstedt, 1982, 1985). This indicates de novo synthesis of linamarin and lotaustralin within the Zygaeninae (Fig. 3). In agreement with previous results, we have observed the presence of linamarin and lotaustralin in several life stages of *Z. transalpiina* (Fig. 5) by LC-MS profiling (Fig. 6). In addition, both linamarin and lotaustralin were detected in the closely related families Heterogynidae, Megalopygidae and Limacodiae (Witthohn and Naumann, 1987a) (Fig. 3). Accordingly, it appears as if the accumulation of linamarin and lotaustralin is a phylogenetically old and monophyletic feature of the Zygaenidae and perhaps also of the Zygaenoidea (Fig. 3).

Linamarin and lotaustralin appear to be sequestered from host plants (Fabaceae, e.g. *L. corniculatus*) in all examined *Zygaena* species (Nahrstedt, 1989). In
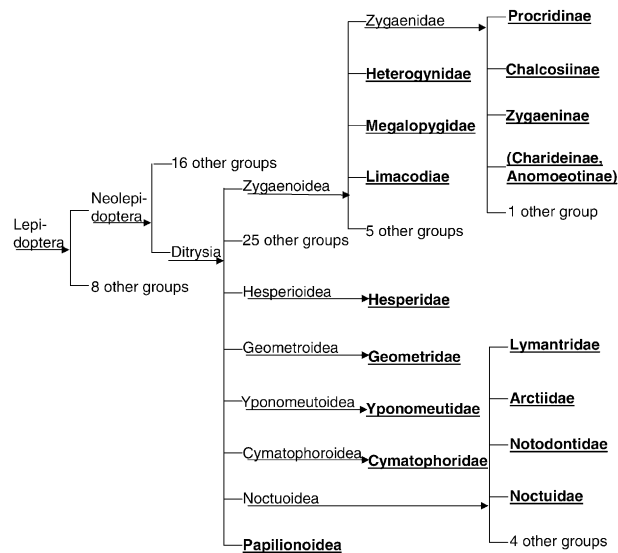

Fig. 3. Simplified schematic evolutionary tree depicting lepidopteran groups. All lepidopteran groups shown in bold and underlined contain linamarin, lotaustralin and β-cyanoalanine in at least one life-stage (Witthohn and Naumann, 1987a).
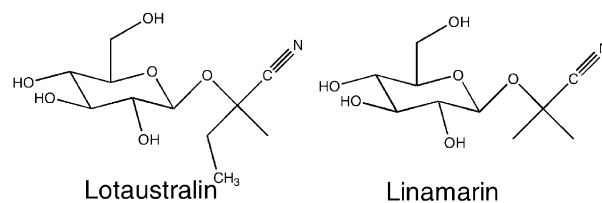

Fig. 4. The cyanogenic glucosides linamarin and lotaustralin derived from valine and isoleucine, respectively.
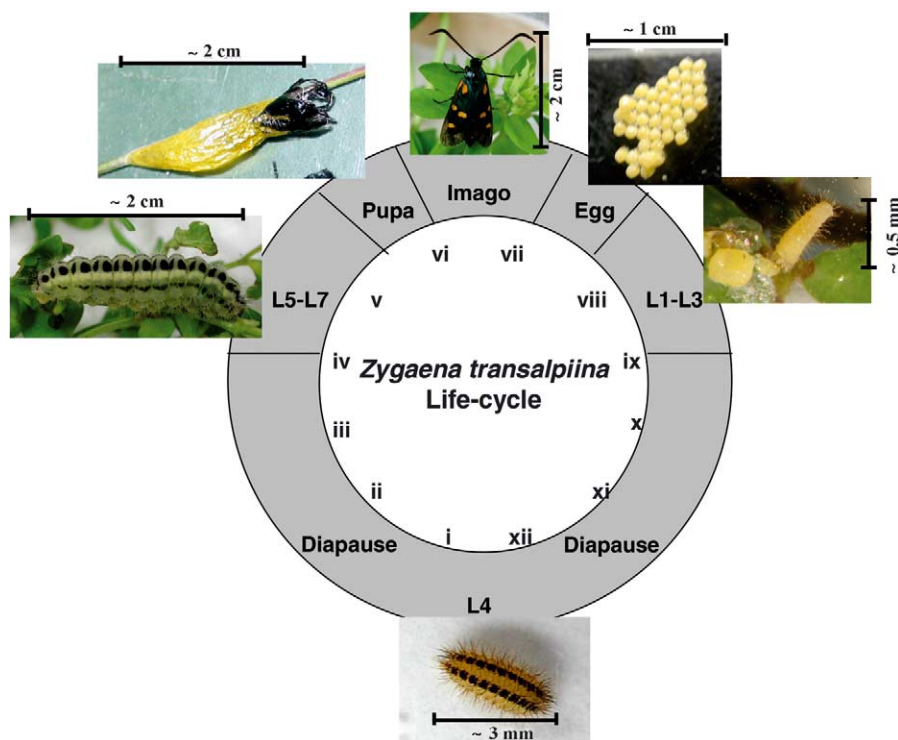
Fig. 5. *Zygaena transalpiina* life-cycle: Eggs are laid in July usually on the larval food-plant. After 1–3 weeks they hatch and the first instar larvae emerge (L1). Molting occurs every 8–10 days and the larvae stop feeding and enter diapause usually in the third, fourth or fifth instar (L3, L4, L5). During diapause, larvae reduce their metabolic turnover by about 70% compared to feeding instars and develop a considerably thicker cuticle. Larvae normally recommence feeding in spring when fresh food plants are available, but diapause may sometimes last for two or more years. The larvae reach maturity in the sixth or seventh instar (L6, L7) and 4–6 weeks after emerging from diapause, they proceed to spin cocoons and pupate. The imago moths emerge from the cocoons 14–30 days later, mate and the females lay eggs. The imagines survive 2–20 days. This life-cycle description applies to most zygaenid species (Naumann et al., 1999).

Z. filipendulae larvae, the amount of lotaustralin was always greater than the amount of linamarin. This probably reflects that lotaustralin is the major CNG in most leaves of one of their food plants *L. corniculatus*. A large natural variation in the linamarin and lotaustralin content in *L. corniculatus* has been reported (Gebrehiwot and Beuselinck, 2001). We confirmed this by analyzing *L. corniculatus* plants collected at three different sites in the greater Copenhagen area (Fig. 7). After pupation and in the subsequent developmental stages, linamarin was the dominant CNG in *Z. filipendulae* and also in other examined Zygaenidae (Davis and Nahrstedt, 1982). Our data obtained with *Z. transalpiina* (Fig. 6) show a more equal distribution of linamarin and lotaustralin in both larvae and imagines, although eggs contained more linamarin and empty pupae contained more lotaustralin. Accordingly, the relative amounts of linamarin and lotaustralin accumulated in Zygaenidae may vary, partly due to the relative amounts present in their diet, and partly because of the ratio generated in the insect by de novo synthesis.

In *Z. trifolii*, only small amounts of CNGs (<1%) were found in the gut and the fat body while the majority was present in haemolymph and integument, including the defensive fluid (Davis and Nahrstedt, 1982; Franzl et al., 1986). In many insects, a large proportion of the accumulated toxic secondary plant products may be excreted or lost with exuviae during the molt. As opposed to this, *Zygaena* larvae are able to retrieve CNGs from the old cuticle (Franzl et al., 1988), since exuviae contain only minute amounts of CNGs (Fig. 6).

5.2. Defensive secretion and cuticular cavities

As a defensive reaction against predators (shrews, hedgehogs, starlings, frogs and carabid beetles), larvae of Zygaenini species may release highly viscous, colorless fluid droplets from cuticular cavities placed on their dorsal side (Fig. 8). Droplets appear on the cuticular surface upon contraction of irritated segments (Franzl and Naumann, 1985). The defensive fluid from *Z. trifolii* has been shown to be composed of linamarin and lotaustralin (7% CNGs) (Fig. 6), β-cyanoalanine (0.3%), proteins (8%, including β-glucosidase) and water (Witthohn and Naumann, 1984). A linamarin:lotaustralin ratio of 1:1 was measured in the defensive secretion whereas that of the haemolymph of
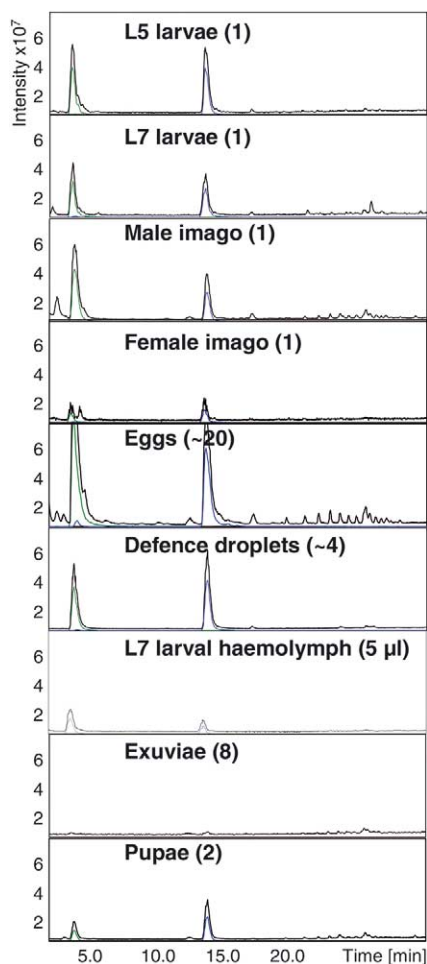
Fig. 6. Linamarin and lotaustralin in *Zygaena transalpiina* stages as monitored by LC–MS. Total ion traces are shown in black and overlaid with selected *m/z* ion traces for linamarin (green) and lotaustralin (blue). The number of specimens used for each sample is shown in parentheses. Due to a large biological variation, the signal intensity shown in the different panels should only be considered semi quantitative.

the *Zygaena* larvae was 19:1 (Franzl et al., 1986). This indicates that lotaustralin is transported more effectively than linamarin, maybe as a result of increased lipid solubility caused by its longer aliphatic side chain (Franzl et al., 1986). Alternatively, the shifted ratio could reflect a slower turnover rate of lotaustralin compared to linamarin in larvae.

Two morphologically different types of cavities have been found in *Z. trifolii* (Fig. 8); the larger cavities release their contents as a response to a slight irritation, whereas the smaller cavities react following severe irritation and release much smaller droplets. Defense droplets may be reabsorbed a few seconds after irritation has stopped. In contrast to most diplopods and chilopods that have specialized cyanogenic glands (Duffey, 1981), there are no gland cells or cuticular ducts leading through the cuticle into the cavities in *Zygaena* larva, and no special morphological adaptation for secretion has been developed in the epidermis (Franzl and Naumann, 1985).

5.3. Metabolism, catabolism and detoxification of cyanogenic glucosides

In plants, the same enzyme system uses valine and isoleucine as precursors for the synthesis of linamarin and lotaustralin, respectively, but with different catalytic efficiencies toward the substrate amino acids. This is reflected in the relative amounts of linamarin and lotaustralin in cassava (Andersen et al., 2000) and in *L. japonicus* (Forslund et al., submitted). In plants, exogenously administered *N*-hydroxyamino acids, aldoximes and nitriles can be incorporated into CNGs (Jones et al., 2000; Møller and Seigler, 1999) These same results are obtained with *Zygaena* species, and suggests that the biosynthetic pathway for CNGs in *Zygaena* is
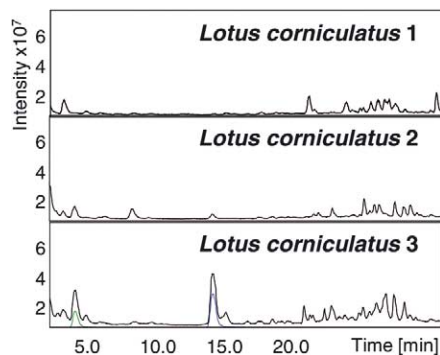

Fig. 7. Linamarin and lotaustralin polymorphism in three different populations of *Lotus corniculatus* from the larger Copenhagen area in Denmark as monitored by LC–MS. Total ion traces are shown in black, overlaid with selected *m/z* ion traces for linamarin (green) and lotaustralin (blue).
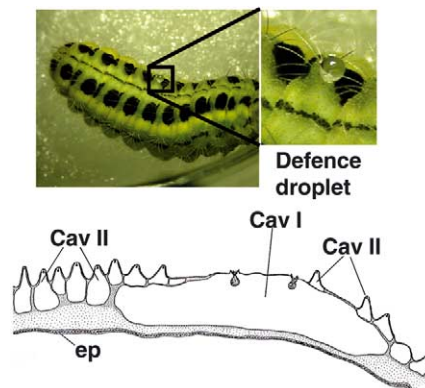

Fig. 8. Last instar larvae of *Zygaena transalpiina* with enlarged defense droplet (top panel). Cuticular cavities from last instar larvae of *Z. trifolii* (lower panel). ep: epidermis; cav I: type I cuticular cavity; cav II: type II cuticular cavity. Adapted from (Naumann et al., 1999).

identical to the pathway in plants (Conn, 1991; Davis and Nahrstedt, 1987; Holzkamp and Nahrstedt, 1994; Nahrstedt, 1996; Wray et al., 1983) (Fig. 1).

β-Glucosidase dependant HCN release has been observed from different life stages of many zygaenid species (Davis and Nahrstedt, 1979; Witthohn and Naumann, 1987b). β-Glucosidase from *Z. trifolii* is a dimer consisting of two supposedly identical 66 kDa subunits. The β-glucosidase exhibits a strong activity towards the endogenous substrates linamarin and lotaustralin, with lotaustralin being a better substrate (Franzl et al., 1989). The *Z. trifolii* β-glucosidase is labile at temperatures above 40 °C and inactivated at a pH below 5 as are β-glucosidases from other insects (*Bombyx mori* and *Trinervitermes trinervoides*). β-Glucosidase activity was found exclusively in haemolymph, which has a pH of 6.2 (Franzl et al., 1989). At pH 6.2, the β-glucosidase is present in an almost inactive state but the enzyme becomes active when pH decreases (Nahrstedt and Müller, 1993). This may point to a situation where stomach acid from a predator will activate the β-glucosidase leading to a rapid and strong release of HCN (Nahrstedt, 1993). Mg^{++} and Ca^{++} ions are inhibitors of the *Zygaena* β-glucosidase. To date, this is the only example of a β-glucosidase which is inhibited by alkaline earth metal ions (Nahrstedt and Müller, 1993).

α-Hydroxynitrile lyase was purified from the haemolymph of *Z. trifolii* and characterized as a FAD-containing enzyme (Müller and Nahrstedt, 1990; Nahrstedt, 1996).

β-Cyanoalanine synthase activity is found in the integument (22%), the fat body (27%) and the gut (12%) of *Zygaena* larvae, with the highest activity of the enzyme in the gut. β-Cyanoalanine synthase detoxifies HCN to β-cyanoalanine, which accumulates in haemolymph (75%) and integument (16%) (Nahrstedt, 1993; Witthohn and Naumann, 1987a). Thus, these insects can easily detoxify HCN. Zygaenidae seem to use the β-cyanoalanine pathway as the sole pathway for detoxification of HCN, since rhodanese (Fig. 1) has not been found in any species containing β-cyanoalanine (Witthohn and Naumann, 1987a).

6. Cyanogenic glucosides in Papilionoidea (butterflies)

6.1. Linamarin and lotaustralin distribution

Imagines of butterflies from the Heliconiinae, Acraeinae, Nymphalinae and Polyommatinae (Papilionoidea) groups (Fig. 3) accumulate linamarin and often lotaustralin in all life stages (Brown and Francini, 1990; Nahrstedt, 1988; Nahrstedt and Davis, 1981; 1983). The butterflies *de novo* synthesize linamarin and lotaustralin from valine and isoleucine, respectively, and because

linamarin and lotaustralin are not present in their larval host plants (Passifloraceae), sequestration cannot occur (Engler et al., 2000; Nahrstedt and Davis, 1983; Wray et al., 1983). The amount of linamarin is higher than that of lotaustralin as also observed in imagines of Zygaenidae, and the CNGs have the same bodily distribution as observed in *Z. trifolii* (Davis and Nahrstedt, 1982; Franzl et al., 1986). The examined butterflies also contain monoglycoside cyclopentenyl cyanogens, probably sequestered from their host plants, at the larval stage (Engler et al., 2000).

6.2. Detoxification, biosynthesis and sequestration of cyanogenic glucosides

In *Heliconius sara* (Papilionoidea) (Fig. 3), monoglycoside cyclopentenyl cyanogens, obtained and sequestered from the host plant are detoxified using a unique enzymatic mechanism not found in the host plants. This mechanism involves substitution of the nitrile group of the cyanohydrin function with a mercapto group (Engler et al., 2000). The reaction mechanism involved has not been elucidated and it remains to be shown whether free cyanide is released during the reaction or whether the nitrile group is transferred to a specific acceptor molecule, like cysteine, as a part of the reaction sequence. The cyanogenic glucoside-derived mercapto compound was not found in the host plant (Engler et al., 2000). This is in contrast to the mechanism proposed for *H. melpomone* (Papilionoidea) (Fig. 3) and *Z. trifolii*. The presence of β-cyanoalanine synthase was demonstrated in *Clossiana euphrosyne* (Papilionoidea) (Witthohn and Naumann, 1987a) (Fig. 3) which confirms that some *Papilionoidea* species detoxify HCN using the same mechanism as Zygaenidae species. Consequently, *H. sara* may either have a unique detoxification system, or insects may use different detoxification systems within the same group.

Eutoptia hegesia (Heliconiinae, Papilionoidea) (Fig. 3) has been hypothesized to de novo synthesize as well as sequester cyclopentenyl glycine derived CNGs from its primary host plant *Turnera ulmifolia* (Schappert and Shore, 1999). De novo biosynthesis was proposed because HCN was measured in larvae reared on acyanogenic plants. Sequestering was proposed because of significantly higher CNG levels in larvae reared on cyanogenic plants compared to siblings on acyanogenic plants (Schappert and Shore, 1999). Accordingly, the ability to both de novo synthesize and sequester the same CNGs appears not to be an exclusive feature for Zygaenidae species.

Within Papilionoidea, de novo biosynthesis of CNGs seem to be less frequent in adults of less advanced genera such as *Agraulus* (Heliconiinae, Papilionoidea), *Dryas* (Heliconiinae, Papilionoidea) and *Cethosia*

(Acraeinae, Papilionoidea) (Fig. 3) compared to the more advanced genera *Heliconius* (Nahrstedt and Davis, 1985). In all Heliconiini (Papilionoidea) and some *Acrea* (Papilionoidea) species (Fig. 3), the ability to synthesize and metabolize CNGs as well as to detoxify HCN may have provided the possibility to change from acyanogenic host plants to the cyanogenic Passifloraceae (Davis and Nahrstedt, 1985). Synthesis and degradation of CNGs in all these butterfly species may have been derived from a common cyanogenic ditrysian ancestor (Fig. 3) and may therefore be homologous to the pathways found in Zygaenidae.

7. Conclusions and perspectives

In plants, cyanogenic glucosides serve as good chemotaxonomic markers for plant relatedness: the more closely related two plant species are, the more similar their cyanogenic glucosides are (Jones, 1988). Within cyanogenic genera, however, acyanogenic species can be found. These are the result of natural mutations that have caused the loss of the ability to produce one or more of the enzymes involved in cyanogenic glucoside biosynthesis and/or in the degradation of CNGs. Secondary loss of enzymes involved in biosynthesis or degradation of CNGs in insects may also be found upon further study. Eventually, it may become apparent that biosynthesis and degradation of CNGs and the ability to detoxify HCN are shared traits between groups of moths and butterflies, and derived from a common cyanogenic ditrysian ancestor (Fig. 3). Studies on a range of ditrysian species are required before this hypothesis can be substantiated or dismissed.

Acyanogenic *L. corniculatus* plants are grazed more heavily by herbivores than cyanogenic *L. corniculatus* plants, but the difference is partly balanced by the ability of acyanogenic plants to grow better than cyanogenic ones (Jones, 1962, 1988). Accordingly, it is probably advantageous for *L. corniculatus* to maintain this polymorphism. This would serve to secure more competitive growth and development in years with no or reduced numbers of herbivores, and to prevail in years with heavy attack from herbivores. Acquisition of an ability to detoxify HCN in some insect species early in their evolution, has offered the opportunity to feed on plants that deter other herbivores, and subsequently exploit the availability of such new host plants to radiate at the expense of otherwise competing species, as seen in moth and butterfly groups. Subsequent acquisition of the ability to sequester CNGs from host plants as a means of detoxification provides such insects with the additional benefits of an improved defense system. Simultaneously, these insects then become more or less dependent on the availability of cyanogenic host plants. In this scenario, it is advantageous for the plant to maintain some acyanogenic genotypes that will not be preferred by the specialized insects. The ability to de novo synthesize CNGs is probably a basic trait within some insect groups. Accordingly, these insects only need cyanogenic host plants to minimize their own biosynthesis of CNGs. Consequently, some moth and butterfly species presumably feed on plants that deter other herbivores without being absolutely dependant on such plants for their own predator defense.

The ability to transfer genes across species using genetic engineering enables the design of plants with an altered qualitative and quantitative content of natural products thereby bypassing millions of years of co-evolution of plants and their herbivores. Transgenic *A. thaliana* plants accumulating the tyrosine-derived CNG dhurrin provided strong evidence that dhurrin serves as a strong feeding deterrent in free choice experiments with the flea beetle *Phyllotreta nemorum* (Tattersall et al., 2001). Similarly, transgenic *Lotus japonicus* plants with altered ratios of linamarin and lotaustralin are available (Forslund et al., submitted) to study sequestering and turnover of linamarin and lotaustralin in *Z. transalpiina*. In this way, chemical warfare between plants and insects can be followed closely through metabolite profiling (LC–MS) and transcript profiling, thereby providing a detailed understanding of the relative importance of complete metabolism, detoxification and sequestering of CNGs.

Many details remain unknown concerning synthesis and degradation of CNGs in insects. Has the ability to de novo synthesize CNGs evolved several times in the course of insect evolution? Or have genes encoding the enzymes involved in biosynthesis, degradation and detoxification of CNGs been horizontally transferred to the insects from e.g. host plants? If the genes have been transferred, did this then happen early in insect evolution or on several occasions? Or is the ability of some insects to synthesize, degrade and detoxify CNGs the result of convergent evolution, where modifications of distantly related genes have enabled recruitment of new desired functions? As more insect species become analyzed and additional information is obtained, answers to some of these questions will be provided. It should be noticed that already with the sparse knowledge currently available, it is obvious that some of the plant enzymes involved in biosynthesis or degradation of cyanogenic glucosides, like the α-hydroxynitrilases, have been recruited by modification of different types of ancestral genes. This may also apply to the arthropods. Valuable new information will appear as more insect genomes, e.g. *Manduca sexta*, *Heliothis virescens*, and *Heliconius erato* (National Human Genome Research Institute), are sequenced.

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