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Transcriptional regulation of *de novo* biosynthesis of cyanogenic glucosides throughout the life-cycle of the burnet moth *Zygaena filipendulae* (Lepidoptera)

Joel Fürstenberg-Hägg [a,b], Mika Zagrobelny [a,b], Carl Erik Olsen [a,b], Kirsten Jørgensen [a,b], Birger Lindberg Møller [a,b], Søren Bak [a,b,*]

[a] *Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*
[b] *VILLUM Research Center "Plant Plasticity", Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*

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ABSTRACT

The six-spotted burnet moth *Zygaena filipendulae* (Lepidoptera) utilize the two cyanogenic glucosides (CNglcs) linamarin and lotaustralin as deterrents against predators throughout the entire life cycle. CNglcs can be hydrolyzed and bioactivated by β-glucosidases, resulting in the release of toxic hydrogen cyanide. CNglcs are retained through metamorphosis, probably involved in mating communication, and transferred during mating from the male to the female as a nuptial gift. CNglcs can be biosynthesized *de novo* by *Z. filipendulae* larvae, but may also be sequestered from their food plant *Lotus corniculatus* (Fabaceae). These two strategies are tightly linked and adjusted according to the CNglc content and composition of the food plant in order to balance CNglc homeostasis in the larva. In this study, the amounts of CNglcs and transcript levels of the biosynthetic genes were monitored in all life-stages and tissues of *Z. filipendulae*. During pupation, transcription of the biosynthetic genes is turned off and the CNglc content slowly declines. In females but not males, transcription of the biosynthetic genes is re-activated at the end of pupation. Eggs and embryos do not biosynthesize CNglcs *de novo*, but are endowed with CNglcs following eclosion of the female. Similarly to larvae, *de novo* biosynthesis in female adults takes place in the integument from which CNglcs are then transported to other organs. This study demonstrates that *Z. filipendulae* has evolved the ability to adjust the production of CNglcs throughout its life-cycle for optimal utilization in defense and possibly other metabolic functions, while at the same time avoiding intoxication.

1. Introduction

An essential driving force in the co-evolution between plants and arthropods is the ability to produce and handle bioactive compounds. While plants produce bioactive specialized compounds for defense, some specialist arthropods have evolved to tolerate these compounds and/or use them in their own defense. Employment of such bioactive compounds requires morphological adaptations and enzymatic machinery for handling and storage without intoxication.

Cyanogenic glucosides (CNglcs) are potent defense compounds found in different lineages in the animal and especially the plant Kingdom of Life. The number of known different CNglcs is rapidly expanding as a result of improved analytical tools (Gleadow and Møller, in press; Neilson et al., 2011). CNglcs are present within pteridophytes (ferns), gymnosperms and angiosperms, while among animals they are only found in some arthropods. CNglcs are multifunctional defense compounds which are deterrent due to their astringent taste (Braekman et al., 1982; Frazer and Rothschild, 1960), but also because they can be hydrolyzed, and thereby

Abbreviations: ab, abdomen; ca, calling organs; cb, corpus bursae; CNglcs, cyanogenic glucosides; eg, eggs; ex, external genitalia; fb, fat body; he, head; ig, internal genitalia; Ile, isoleucine; in, ingluvie; P450s, cytochromes P450; Pg, Petersens glands; rb, rectal bladder; sg, sebacceous glands; sp, spermatophore; te, testes; th, thorax; UGT, UDP-dependent glycosyltransferase; Val, valine; wi, wings.

* Corresponding author. Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark. Tel.: +45 35333346.
E-mail address: bak@plen.ku.dk (S. Bak).

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bioactivated, by β-glucosidases resulting in the formation of cytotoxic aldehydes and ketones (Zagrobelny et al., 2004), as well as highly toxic hydrogen cyanide (HCN) (Way, 1984). Some arthropods have evolved to tolerate CNglcs and use them as phagostimulants and/or as oviposition cues (Ballhorn et al., 2010; Gleadow and Woodrow, 2002; Jones, 1988; Zagrobelny et al., 2007b). In many cases, these specialized arthropods also utilize CNglcs in their own defense against predators (Zagrobelny et al., 2008).

In both plants and arthropods, CNglcs are biosynthesized *de novo* from amino acids by pathways that have evolved convergently (Jensen et al., 2011). These pathways include similar intermediates and the same enzymatic steps, in both cases carried out by two multifunctional cytochromes P450 (P450s) and a UDP-dependent glycosyltransferase (UGT) (Fig. 1) (Bak et al., 2006; Jensen et al., 2011; Jørgensen et al., 2005; Takos et al., 2011). The first P450 catalyzes the signature step, the transformation of the amino acid into the corresponding oxime. The second P450 catalyzes the subsequent transformation of the oxime into a labile α-hydroxynitrile (cyanohydrin). As the last step in the biosynthetic pathway, the α-hydroxynitrile is converted into a cyanogenic glucoside by a UGT.

The six-spotted burnet moth *Zygaena filipendulae* (Zygaenoidea, Lepidoptera) uses the CNglcs linamarin and lotaustralin in its defense against predators (Rammert, 1985, 1992; Wiklund and Järvi, 1982). The CNglcs are acquired at the larval stages both through sequestration (uptake and accumulation (Duffey, 1980)) from their food plant *Lotus corniculatus* (Fabaceae) and by *de novo* biosynthesis (Davis and Nahrstedt, 1987; Holzkamp and Nahrstedt, 1994; Nahrstedt and Davis, 1986; Wray et al., 1983). In *Z. filipendulae*, the two CNglcs linamarin and lotaustralin are biosynthesized *de novo* from the amino acids valine (Val) and isoleucine (Ile), respectively, (Wray et al., 1983) by the enzymes CYP405A2, CYP332A3, and UGT33A1 (Fig. 1) (Jensen et al., 2011). The total CNglc levels and the ratio between linamarin and lotaustralin are tightly regulated in *Z. filipendulae* (Zagrobelny et al., 2007b).

Z. filipendulae undergoes complete metamorphosis (holometabolism) which includes the embryonic, larval, pupal and adult developmental stages (Figure S1). Eggs are laid around midsummer, usually on the larval food plant, upon which they hatch 1–3 weeks later, yielding the first of seven instars (L1 larva). Molting occurs every 8–10 days. The fourth instar (L4) is reached by the end of summer, after which the larva enters a non-feeding diapause period. The diapause ends when the temperatures rise in the spring at which point the larva resumes feeding. After 4–6 weeks the seventh instar (L7) larva reaches maturity, spins a cocoon and pupates. Pupation lasts 14–20 days, after which the adult emerge (eclosion) (Naumann et al., 1999; Zagrobelny et al., 2008). Unfolding, drying and hardening of the wings take from a couple of hours up to a day, after which the female begins to call for males and the male begins to search for females. In the laboratory, where pairs of males and females are placed in the same container, courtship and mating usually occurs within one day after eclosion, while the timing for courtship and mating in the field depends on the availability of adults of both sexes in the same area as well as the weather conditions. Mating lasts 16–24 h (Zagrobelny et al., 2013), and eggs are laid shortly after mating. The adult lifespan is up to 3 weeks in both males and females.

Linamarin and lotaustralin are present throughout the entire life cycle of *Z. filipendulae* (Davis and Nahrstedt, 1982; Zagrobelny et al., 2008), and function as defense compounds for larvae (Rammert, 1985) and adults (Wiklund and Järvi, 1982). Adults may also utilize CNglcs and/or derivatives thereof in mating communication (Zagrobelny et al., 2008, 2007a), and during mating the male transfers CNglcs to the female as part of a nuptial gift (Zagrobelny et al., 2007a, 2013, 2014). In addition, CNglcs could function as a precopulatory offering, enabling the female to access how much CNglcs the male possesses and would be able to contribute. Accordingly, males that contain low amounts of CNglcs are generally rejected by the females (Zagrobelny et al., 2007a). It has also been hypothesized that CNglcs are used by insects as a source of reduced nitrogen. The amounts and ratios of linamarin and lotaustralin vary through the developmental stages in *Zygaena* (Zagrobelny et al., 2008). The eggs contain very high amounts of the two CNglcs although it was not known if they were biosynthesized *de novo* by the embryo and/or transferred from the adult female (Zagrobelny et al., 2014). In addition, the timing of these events is poorly understood. Linamarin and lotaustralin accumulate steadily in larvae and reach maximum levels in the seventh instar (L7), in which linamarin and lotaustralin are present in a 1:1 ratio (Zagrobelny et al., 2007b). Linamarin and lotaustralin are retained in adults (Zagrobelny et al., 2014), but the amounts are reduced by ~55% compared to the amounts in larvae, indicating turnover during pupation (Zagrobelny et al., 2008, 2014). Also, the linamarin to lotaustralin ratio is higher (3:1) in adults compared to larvae (Zagrobelny et al., 2007a). The biological reasons for the variation in CNglc ratios during the life cycle remain unknown.

In this study we demonstrate that in both sexes *de novo* biosynthesis of linamarin and lotaustralin is turned off at the start of the pupation period. In adults, *de novo* biosynthesis of linamarin and lotaustralin is sex dependent, since at the end of pupation it is turned on again in females, but not in males. Laid eggs contain high amounts of CNglcs which are transferred after eclosion of the females. The demonstrated *de novo* biosynthesis in females prior to eclosion enables them to replenish their linamarin and lotaustralin reserves after transfer to their eggs. The *de novo* biosynthesis of CNglcs is localized to the abdominal integument and fat body of the female adult.

2. Materials and methods

2.1. Biological material

Z. filipendulae larvae and cocoons were collected from a natural population in the greater Copenhagen area in 2011 and 2012 (N 55° 38.077′, E 12° 15.748′) (Zagrobelny et al., 2007b). Since *de novo* biosynthesis in *Z. filipendulae* larvae is affected by the linamarin and lotaustralin content present in their food plant (*L. corniculatus*) (Zagrobelny et al., 2007b), larval food plants with similar and high

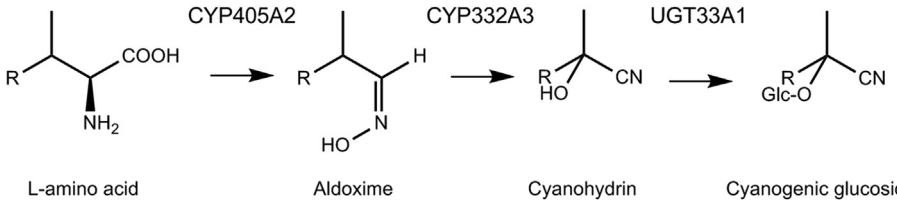

Fig. 1. *De novo* biosynthesis of cyanogenic glucosides in *Z. filipendulae*. R represents a methyl group in linamarin and an ethyl group in lotaustralin.