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Metabolism, excretion and avoidance of cyanogenic glucosides in insects with different feeding specialisations

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ABSTRACT

Cyanogenic glucosides (CNglcs) are widespread plant defence compounds releasing toxic hydrogen cyanide when hydrolysed by specific β-glucosidases after plant tissue damage. In contrast to specialist herbivores that have mechanisms to avoid toxicity from CNglcs, it is generally assumed that non-adapted herbivores are negatively affected by CNglcs. Recent evidence, however, implies that the defence potential of CNglcs towards herbivores may not be as effective as previously anticipated. Here, performance, metabolism and excretion products of insects not adapted to CNglcs were analysed, including species with different degrees of dietary specialisation (generalists, specialists) and different feeding modes (leaf-snipping lepidopterans, piercing-sucking aphids). Insects were reared either on cyanogenic or acyanogenic plants or on an artificial cyanogenic diet. Lepidopteran generalists (*Spodoptera littoralis*, *Spodoptera exigua, Mamestra brassicae*) were compared to lepidopteran glucosinolate-specialists (*Pieris rapae, Pieris brassicae, Plutella xylostella*), and a generalist aphid (*Myzus persicae*) was compared to an aphid glucosinolate-specialist (*Lipaphis erysimi*). All insects were tolerant to cyanogenic plants; in lepidopterans tolerance was mainly due to excretion of intact CNglcs. The two *Pieris* species furthermore metabolized aromatic CNglcs to amino acid conjugates (Cys, Gly, Ser) and derivatives of these, which is similar to the metabolism of benzylglucosinolates in these species. Aphid species avoided uptake of CNglcs during feeding. Our results imply that non-adapted insects tolerate plant CNglcs either by keeping them intact for excretion, metabolizing them, or avoiding uptake.

1. Introduction

Although plants have evolved a high diversity of chemical defences, they are frequently consumed by insect herbivores (Després et al., 2007; Heckel, 2014; Heidel-Fischer and Vogel, 2015). Herbivore species are usually classified based on the degree of dietary specialisation: while generalists feed on numerous plant species, specialists feed on a narrow range of related plant species and often possess adaptations to the defence chemistry of the host (Ali and Agrawal, 2012; Fürstenberg-Hägg et al., 2013). Cyanogenic glucosides (CNglcs) constitute an illustrative example of plant chemical defence compounds which on one hand are overcome by specialists (Pentzold et al., 2014a; Zagrobelny et al., 2014), but on the other hand successfully deter or intoxicate non-adapted herbivores (Ballhorn et al., 2005; Hay-Roe et al., 2011; Tattersall et al., 2001). CNglcs as typical phytoanticipins are stored in vacuoles and kept compartmentally separated from the specific β-glucosidases that hydrolyse them after plant tissue damage resulting in release of toxic HCN (Morant et al., 2008; Pentzold et al., 2014b; Zagrobelny et al., 2008). However, accumulating evidence implies that the defence potential of CNglcs towards herbivores may sometimes be

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limited against both insect generalists or insects not specifically adapted to CNglcs (Ferreira et al., 1997; Fitzgerald, 2008; Gleadow and Woodrow, 2002; Marana et al., 2000; Scriber, 1978; Shlichta et al., 2014; Stauber et al., 2012).

From the plant's perspective, the rate of cyanogenesis (HCN emission) during herbivory is crucial for defence, but it depends on the concentration of CNglcs, the activity of the specific β-glucosidase and the amount of tissue damage by feeding herbivores (Ballhorn et al., 2010, 2005; Gleadow and Woodrow, 2002; Krothapalli et al., 2013; Pentzold et al., 2014a). Thus, for specialist insects that sequester CNglcs it seems beneficial to avoid hydrolysis of plant CNglcs. The lepidopteran *Zygaena filipendulae* larvae for example combines several strategies during feeding and digestion such as a leaf-snipping feeding mode with minimal tissue damage, a highly alkaline midgut lumen avoiding plant β-glucosidase activity, and endogenous β-glucosidases in the digestive tract without activity towards the CNglcs present in the food plant (Pentzold et al., 2014a). Thus, CNglcs stay intact in the gut, enabling these larvae to sequester intact CNglcs (Zagrobelny et al., 2014). In contrast to specialists, generalists or non-adapted insects may avoid contact, ingestion or accumulation of CNglcs in their body, inhibit HCN liberation or metabolize CNglcs.

Similar to CNglcs and β-glucosidases, glucosinolates and myrosinases also constitute a two-component defence system (Fig. 1): the two components are separated in the undamaged plant cell or tissue, but are mixed after plant tissue damage resulting in the enzymatic release of toxic aglucones such as isothiocyanates (ITCs) (Halkier and Gershenzon, 2006; Winde and Wittstock, 2011). In parallel to CNglcs, some insect herbivores are able to overcome the glucosinolate-myrosinase system. This includes glucosinolate-specialists such as the lepidopteran larvae *Pieris rapae* and *Plutella xylostella* that enzymatically either redirect glucosinolate hydrolysis towards less toxic nitriles (Wittstock et al., 2004) or avoid glucosinolate hydrolysis (Ratzka et al., 2002), and the aphid *Lipaphis erysimi* that sequester intact glucosinolates (Bridges et al., 2002). Moreover, the lepidopteran generalists *Mamestra brassicae* and *Spodoptera littoralis* disarm toxic ITCs by conjugation with glutathione (Schramm et al., 2012).

This study takes advantage of the similarity in activation between CNglcs and glucosinolates and tests whether and how CNglcs are metabolised in insect species that are *not* specialised on CNglcs, including generalists as well as specialists adapted to glucosinolates. The study also includes species with different feeding modes, i.e. leaf-snipping lepidopterans and piercing-sucking aphids. It was analysed whether CNglcs or their degradation products were present in the body or frass from insects fed either cyanogenic plants, acyanogenic plants, or an artificial diet containing a cyanogenic di-glucoside as a comparison to the cyanogenic mono-glucosides. Our findings suggest that a variety of insect herbivore species tolerate plant CNglcs during feeding and digestion: they either excrete intact CNglcs, metabolize CNglcs to conjugate breakdown products with amino acids, or avoid CNglc-uptake altogether.

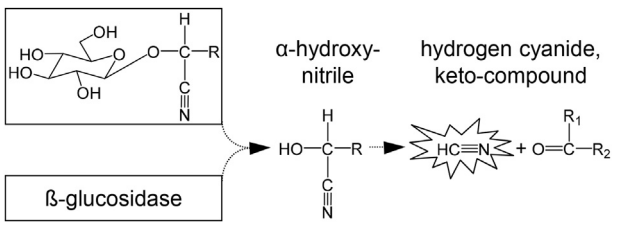

Fig. 1. CNglcs and β-glucosidases constitute a two-component defence system. In eudicotyledenous plants, CNglcs are stored in the vacuole, whereas specific β-glucosidases are localized in the apoplast (Morant et al., 2008). After tissue damage by e.g. herbivory both components come into contact, which results in enzymatic hydrolysis of CNglcs and release of toxic HCN. Degradation of the α-hydroxynitrile can be spontaneous or due to α-hydroxynitrile lyase activity. R – aliphatic or aromatic side chain.

2. Materials and methods

2.1. Biological material and assays

Third instar larvae of the lepidopteran generalists *M. brassicae* (N = 4) and *Spodoptera exigua* (N = 10), and the generalist aphid *Myzus persicae* (N~50 adults) as well as the glucosinolate-specialist lepidopterans *P. rapae* (N = 4), *P. brassicae* (N = 6), *P. xylostella* (N = 10) and the aphid *L. erysimi* (N~50 adults) were fed either transgenic *Arabidopsis thaliana* leaves containing the aromatic CNglc dhurrin (Tattersall et al., 2001) or acyanogenic *A. thaliana* leaves (Col-0). The transgenic *A. thaliana* plant has an endogenous β-glucosidase with some dhurrin hydrolysing activity and thus, after tissue damage, release HCN up to ~2 μmol/gfw (Tattersall et al., 2001). Three of the above mentioned insect species (*P. rapae, P. brassicae*, *P. xylostella*) were also fed a wheat germ based artificial diet containing the aromatic cyanogenic di-glucoside amygdalin, which is derived from the mono-glucoside prunasin. A 10% (w/v in H_2O) solution of amygdalin (Sigma 10050, >97% purity, with no prunasin contamination) was applied to the artificial diet at a volume of 10 μl per g diet (soaking). The larvae were allowed to feed on the soaked diet for 3–4 days. The insects were starved overnight before a feeding experiment and fed a non-cyanogenic diet or Col-0 leaves after the experiment to potentially empty their gut of CNglcs. Insect development and potential signs of intoxication were carefully observed. Insects and frass were frozen in liquid nitrogen and kept at −80 °C until extracted for LC-MS analyses.

Larvae of the lepidopteran generalist *S. littoralis* (3rd instar, N = 14) were fed leaves of *Lotus japonicus* plants either containing (MG-20) or lacking (*cyd1*) the aliphatic CNglcs lotaustralin and linamarin (Takos et al., 2010). MG-20 plants release high levels of HCN upon tissue damage due to specific endogenous β-glucosidase activity (Takos et al., 2010). MG-20 leaves also contain the rhodiocyanosides D and A, which are β- and γ-hydroxynitrile glucosides, whereas CNglcs are α-hydroxynitrile glucosides. Thus, in contrast to CNglcs, rhodiocyanosides do not release HCN after hydrolysis by β-glucosidase activity (Bjarnholt et al., 2008). Larvae were reared for 8 days and weight gain was measured daily. All feeding experiments were no-choice diets. Mean values and standard error of the mean (s.e.m.) for individual weight gain per day were calculated and tested for significant differences using two-tailed Student's t-test (SigmaPlot 12.0, Systat Software). Differences were considered statistically significant at $P < 0.05$. Seventh instar larvae of the lepidopteran specialist *Z. filipendulae* (N = 3) where fed *Lotus corniculatus* leaves (containing endogenous lotaustralin and linamarin) painted with 5 μl 10 mM prunasin each. Larvae were starved for 24 h after the whole leaf was consumed to empty the gut. Larvae, frass and leaves where frozen in liquid nitrogen and kept at −80 °C until extracted for LC-MS analyses.

2.2. LC-MS/MS

Metabolites from frozen insects (1 individual per sample) and frass (20–40 mg) were extracted by maceration in 55% MeOH containing 0.1% HCOOH using a pestle and mortar; the macerate was subsequently passed through an Anopore 0.45 μm filter (Whatman). Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Santa Clara, CA, US) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Germany). Chromatographic separation was done using a Zorbax