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Rapid incorporation of glucosinolates as a strategy used by a herbivore to prevent activation by myrosinases

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ABSTRACT

Various plants have a binary defence system that consists of a substrate and a glucosidase, which is activated upon tissue disruption thereby forming reactive hydrolysis products. Insects feeding on such plants have to overcome this binary defence system or prevent the activation. In this study, we investigated the strategy used by a herbivore to deal with such binary defence. We studied, how the larvae of the sawfly *Athalia rosae* (Hymenoptera: Tenthredinidae) circumvent the activation of glucosinolates by myrosinase enzymes, which are found in their Brassicaceae host plants. Myrosinase activities were low in the front part of the larval gut but activities increased over the gut passage. In contrast, the glucosinolates were only highly concentrated in the first gut part and were rapidly incorporated into the haemolymph before the food reached the second half of the gut. Thus, the uptake and concentration of glucosinolates, i.e., sequestration, must occur in the front part of the gut. Using Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging (MALDI-MSI), we could demonstrate that the incorporated glucosinolate sinalbin circulates in the haemolymph where it accumulates around the Malpighian tubules. This study highlights the pivotal role of the gut of an adapted herbivore as a regulatory functional organ to cope with plant toxins. MALDI-MSI turned out as a highly useful technique to visualise glucosinolates in a herbivore, which has to deal with plants exhibiting a binary defence system, and may be applied to follow the fate of plant metabolites in other insect species in the future.

1. Introduction

While plants exploit different strategies to fend off herbivores, generalist and specialist insects have found ways to attack and feed on plants due to various behavioural or physiological adaptations. In plant species of the family Brassicaceae, a well known and widely studied chemical defence system occurs comprising two spatially separated partners, glucosinolates and their hydrolysing enzymes, the myrosinases (Agerbirk and Olsen, 2012). The glucosinolate molecule consists of a β-thioglucose moiety, a sulphonated oxime moiety, and a variable side chain derived from different amino acid precursors (Mithen, 2001) (Fig. 1). The myrosinases are thioglucosidases, whose pH optima are often slightly acidic ranging between pH 4 and 7 (Bones and Rossiter, 1996; Travers-Martin et al., 2008). Upon tissue damage, glucosinolates are readily hydrolysed by plant myrosinases yielding unstable aglucones that further react to various products, namely isothiocyanates, thiocyanates, nitriles, epithionitriles, or oxazolidine-2-thiones, depending on the pH value, availability of ferrous ions, specifier proteins, and other factors (Hopkins et al., 2009; Agerbirk and Olsen, 2012). These reactive products can have detrimental effects on different herbivores and pathogens (Rask et al., 2000; Agrawal and Kurashige, 2003; Wittstock et al., 2003; Hopkins et al., 2009). Avoidance of this binary defence system by herbivores can be realised by a feeding mode in which plant tissues are not disrupted or by alternative physiological strategies (Fig. 1). The diversity of adaptations to the same plant defence system has intrigued many researchers studying plant–insect interactions (Renwick, 2002; Gershenzon and Müller, 2009).

By sucking only from the phloem, aphids can circumvent the disruption of cells and thus the hydrolysis of glucosinolates. For example, the generalist aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) excretes ingested glucosinolates with the honeydew (Weber et al., 1986). Alternatively, some specialist aphids, such as *Brevicoryne* brassicae (L.) and *Lipaphis erysimi* (Kaltenbach) (Hemiptera: Aphididae), as well as the harlequin bug, *Murgantia histrionica* Hahn (Hemiptera: Pentatomidae), take up the glucosinolates and sequester them into their haemolymph (MacGibbon and

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Beuzenberg, 1978; Aliabadi et al., 2002; Bridges et al., 2002). Insects that cause tissue damage due to a chewing feeding mode either conjugate the isothiocyanates, like several lepidopterans (Schramm et al., 2012), or redirect the formation of isothiocyanates. Caterpillars of *Pieris rapae* (L.) (Lepidotera: Pieridae) express a nitrile specifier protein in their guts that converts the aglucones to nitriles (Wittstock et al., 2004) which in turn are further metabolised and detoxified (Agerbirk et al., 2006; Stauber et al., 2012). Other herbivores have a sulfatase that converts glucosinolates into desulfoglucosinolates, which are excreted (Ratzka et al., 2002; Falk and Gershenzon, 2007). Although larvae of the sawfly *Athalia rosae* L. (Hymenoptera, Tenthredinidae) and the beetle *Phyllotreta striolata* Fab. (Coleoptera: Chrysomelidae) also have a chewing feeding mode, they are able to sequester glucosinolates into their haemolymph before hydrolysis occurs (Müller et al., 2001; Beran et al., 2014). In the sawfly larvae, at some point the glucosinolates are converted via desulfoglucosinolates to desulfoglucosinolate-3-sulfates, which are then excreted (Opitz et al., 2011). It is still unclear how insects with a chewing feeding mode circumvent the hydrolysis of glucosinolates before incorporation into the haemolymph and how fast the glucosinolates are sequestered.

More than a decade ago, matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry imaging (MSI) was established to analyse the distribution of metabolites in thin-sections of organisms (Spengler et al., 1994; Caprioli et al., 1997). This technique has been, for example, applied to localise glucosinolates in leaves, flowers, and siliques of *Arabidopsis thaliana* (L.) Heynh., where a non-uniform distribution was revealed (Shroff et al., 2008; Sarsby et al., 2012). However, to our knowledge MALDI-MSI has so far not been applied to analyse herbivores feeding on glucosinolate-containing plants. The high sensitivity of MALDI-MSI for locally distributed molecules and the possibility to analyse a wide range of molecules in tissues (Gagnon et al., 2012) encouraged us to employ this technique to localise glucosinolates in an insect that sequesters these metabolites.

In the present study, we investigated potential mechanisms that may underlie the uptake of intact glucosinolates by a chewing herbivore, studying the localisation of glucosinolates and activity of myrosinases in different body parts of *A. rosae* after feeding on *Sinapis alba* L. plants. Furthermore, we studied the speed of sinalbin (*p*-hydroxybenzyl glucosinolate) uptake, the dominant glucosinolate of *S. alba*. We demonstrate that glucosinolates are transported into the haemolymph very rapidly in the front part of the gut of *A. rosae* larvae, before myrosinases become more active in successive gut parts. Different ionic intensities obtained by MALDI-MSI for deprotonated sinalbin in the larvae revealed that sequestered glucosinolates circulate in the haemolymph where they accumulate around the Malpighian tubules before they are metabolised. The gut thus plays a pivotal role in overcoming the binary defence of the host plants.

2. Materials and methods

2.1. Plants and insects

Seeds of *S. alba* var. Silenda (obtained from Raiffeisen GmbH & Co. KG, Bielefeld) were planted in 12 cm diameter pots in a greenhouse (20 °C, 70% r.h., L16:D8). Seedlings were fertilised with diluted Aglukon fertiliser (GmbH & Co. KG; 2 ml fertiliser/l water) weekly until they were six weeks old. The dominant glucosinolate in *S. alba* is sinalbin (*p*-hydroxybenzyl glucosinolate) which contributes more than 60% to the total glucosinolate concentration. For myrosinase and glucosinolate analyses of *S. alba*, middle-aged (third) leaves of undamaged plants were used. Adults of *A. rosae* were collected at field sites near Bielefeld University (Germany) and reared for several generations in a greenhouse (20 °C, L16:D8) on plants of *S. alba* and *Brassica rapa* L. ssp. *chinensis* (var. Michihili). For pH measurements (*2.2.*) and myrosinase and glucosinolate analyses (*2.3.*) of larval tissues, third-instar larvae were fed exclusively on *S. alba* plants until they reached the fifth instar and then used for analyses. For dissection, larvae were placed on glass slides on ice and opened longitudinally from the dorsal side using microscissors. Fat bodies were removed using fine forceps. Guts were washed two times in ice-cold deionised water to clean them from haemolymph and then dried on filter paper.

2.2. pH measurements of gut and plant tissues and pH optimum of plant myrosinases

Two guts of *A. rosae* larvae ($n = 3$ replicates) as well as two leaf discs from middle-aged leaves of *S. alba* (1.7 cm diameter, $n = 5$) were homogenised in 300 µl deionised water (pH 6.5) and the pH

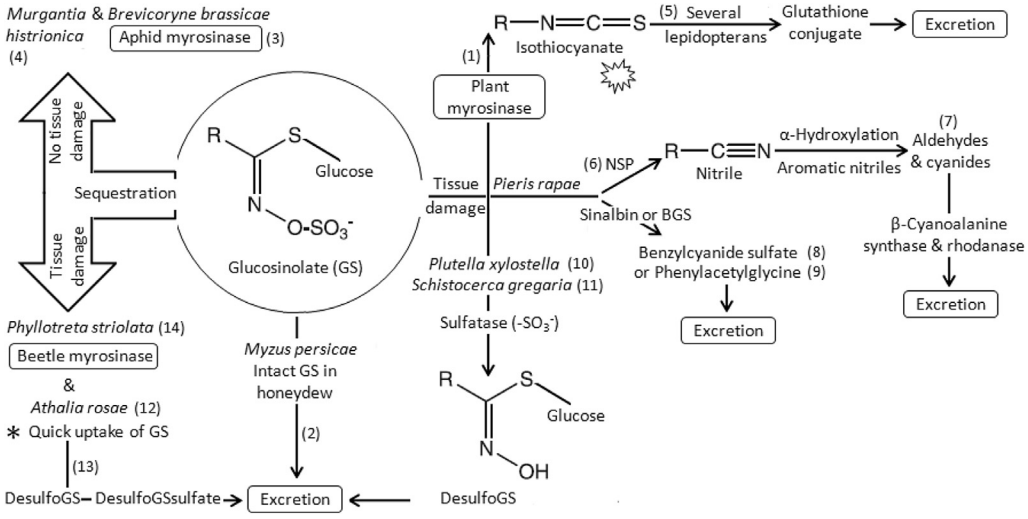

Fig. 1. Different strategies used by insect herbivores to deal with the glucosinolate-myrosinase system, based on (1) Agerbirk and Olsen, 2012; (2) Weber et al, 1986; (3) Bridges et al., 2002; (4) Aliabadi et al., 2002; (5) Schramm et al., 2012; (6) Wittstock et al., 2004; (7) Stauber et al., 2012; (8) Agerbirk et al., 2006; (9) Vergara et al., 2006; (10) Ratzka et al., 2002; (11) Falk and Gershenzon, 2007; (12) Müller et al., 2001; (13) Opitz et al., 2011; (14) Beran et al., 2014. The asterisk indicates the strategy investigated in this study. NSP, nitrile specifier protein; BGS, benzyl glucosinolate. For details, see introduction.