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Spatial organization of the glucosinolate–myrosinase system in brassica specialist aphids is similar to that of the host plant

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Secondary metabolites are important in plant defence against pests and diseases. Similarly, insects can use plant secondary metabolites in defence and, in some cases, synthesize their own products. The paper describes how two specialist brassica feeders, *Brevicoryne brassicae* (cabbage aphid) and *Lipaphis erysimi* (turnip aphid) can sequester glucosinolates (thioglucosides) from their host plants, yet avoid the generation of toxic degradation products by compartmentalizing myrosinase (thioglucosidase) into crystalline microbodies. We propose that death, or damage, to the insect by predators or disease causes disruption of compartmentalized myrosinase, which results in the release of isothiocyanate that acts as a synergist for the alarm pheromone *E*-β-farnesene.

Keywords: thioglucosides (glucosinolates); thioglucoside glucohydrolase (EC 3.2.1 myrosinase); *Brevicoryne brassicae*; aphid; immunolocalization

1. INTRODUCTION

A characteristic of cruciferous plants is the presence of thioglucosides, a class of secondary metabolite commonly known as glucosinolates, which, on contact with myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1), are hydrolysed to biologically active products (figure 1). Hydrolysis can occur either as part of a natural physiological process or by tissue disruption caused by a pest or pathogen. In germinating seeds, myrosinase is present in specialized cells known as myrosin cells, whereas the glucosinolate is held separately in aleurone-like cells (Kelly *et al.* 1998). Recently, in *Arabidopsis*, glucosinolate-specific cells have been directly identified in the flower stalk (Koroleva *et al.* 2000). In *Brassica juncea* myrosinase is present in phloem parenchyma cells (figure 2*a*,*c*) and it was thought that, in the developing plant both glucosinolate and myrosinase were present in myrosin cells. This system has been called a 'mustard oil bomb' (Luthy & Matile 1984) as it constitutes a defence system against pests and diseases. However, recent evidence (Kelly *et al.* 1998) suggests that this may not be the case and glucosinolate and myrosinase may be held in separate cells.

Amongst all the aphids examined for myrosinase (MacGibbon & Allison 1968), only the crucifer specialists *Brevicoryne brassicae* and *Lipaphis erysimi* possessed activity, although some other polyphagous aphids, such as *Myzus persicae*, can also feed on crucifers. Myrosinase was restricted to the head and thorax regions, and no specific internal organ could be associated with the activity (MacGibbon & Beuzenberg 1978). It would appear that a potential role for the myrosinase–glucosinolate system in the cruciferous specialists is in generating isothiocyanates, which act as synergists for the alarm pheromone *E*-β-farnesene (Dawson *et al.* 1987), which communicates a warning to other members of a developing colony. We set out to explore the organization and potential role of the myrosinase–glucosinolate system in both the cruciferous specialists *B. brassicae* and *L. erysimi*.

2. MATERIAL AND METHODS

(a) *Light microscopy: aldehyde fixation–alcohol dehydration—aphids*

Adult aphids were bisected at the juncture of thorax and abdomen, and were vacuum infiltrated with 2.5% glutaraldehyde (v/v) in 50 mM sodium phosphate buffer (pH 7.2) at 4 °C overnight, washed in 50 mM sodium phosphate buffer (pH 7.2), partially dehydrated in an ethanol series (to 70% EtOH v/v) and embedded in medium-grade LR white resin (Agar Scientific, Stansted, Essex, UK).

(b) *Light microscopy: aldehyde fixation–alcohol dehydration—plant material*

Plant material was dissected in, and vacuum infiltrated with, 4% paraformaldehyde, 2% glutaraldehyde (v/v) in 50 mM sodium phosphate buffer (pH 7.2) at 4 °C overnight, washed in 50 mM sodium phosphate buffer (pH 7.2), dehydrated in an ethanol series and embedded in medium-grade LR white resin (Agar Scientific).

(c) *Light microscopy: cryofixation–freeze substitution*

Adult aphids were bisected at the juncture of thorax and abdomen, mounted onto a nylon filament using exuded

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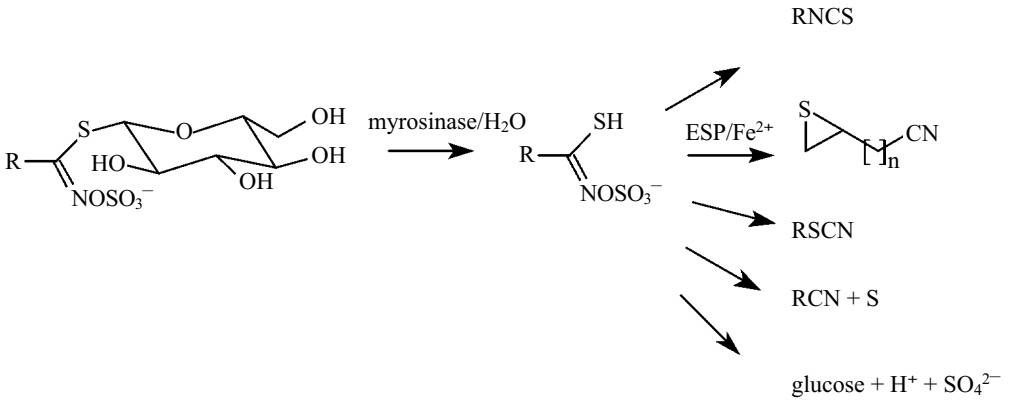

Figure 1. The enzymic hydrolysis of glucosinolates.

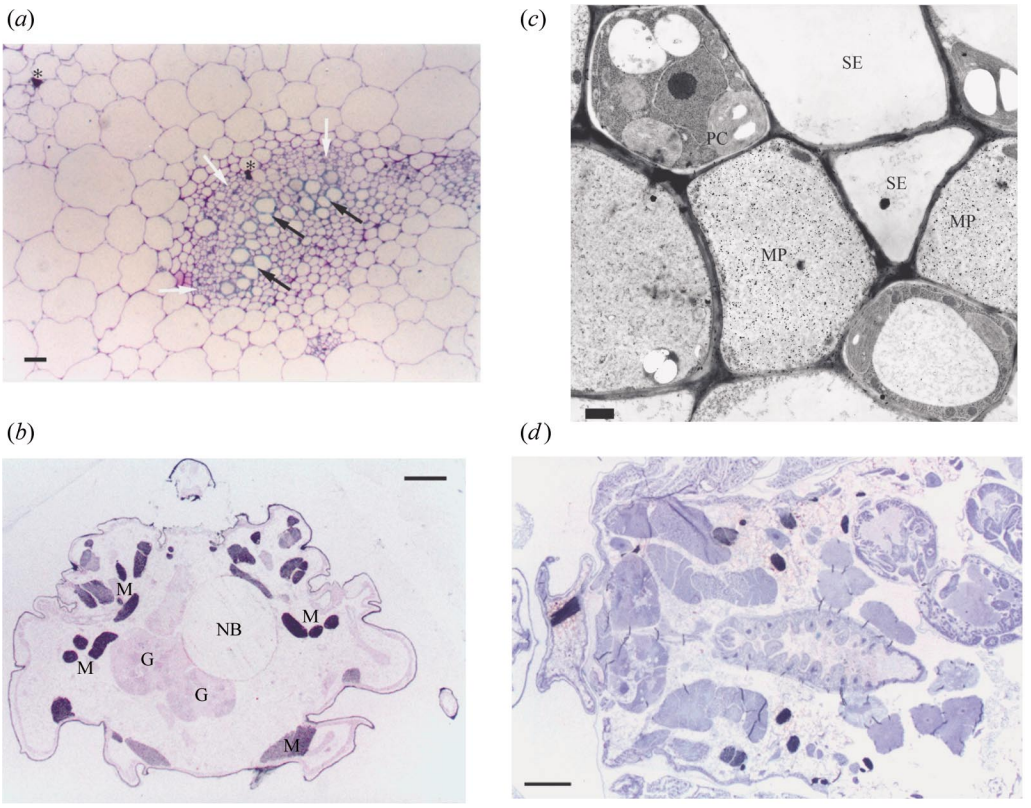

Figure 2. Light microscopy of a transverse section through the proximal region of leaf petiole of *Brassica juncea*, and transverse and longitudinal sections through *Brevicoryne brassicae* stained with anti-myrosinase antibodies. (*a*) This section shows one of the vascular bundles. Anti-myrosinase antibody (polyclonal raised to pure *Sinapis alba* myrosinase, K089; Bones & Thangstad 1991) treatment, followed by enhancement with nucleated silver, shows myrosin cells within the mesophyll tissue and the phloem tissue of the vascular bundles. Asterisks, myrosin cells; white arrows, phloem region; black arrows, xylem elements; scale bar, 100 μm. (*b*) Light microscopy of a transverse section through the abdomen (cryofixation) of *B. brassicae*; anti-aphid myrosinase-antibody-treated section. Following incubation with serum and antisera, sections were stained with goat anti-rabbit (10 nm gold conjugate) antibody and enhanced with nucleated silver; NB, nylon bristle used to support specimen during cryofixation; M, muscle; G, gut; scale bar, 100 μm. (*c*) Transmission electron micrograph of immunolabelled myrosinase on a transverse section through the proximal region of the leaf petiole of *B. juncea;* labelled myrosin cells (MP), phloem companion cells (PC) and phloem sieve elements (SE) are visible; scale bar, 1 μm. (*d*) Light microscopy of a longitudinal section through the head, thorax and first abdominal segments of *B. brassicae*; anti-aphid myrosinase-antibody-treated section. Following incubation with serum and antisera, sections were stained with goat anti-rabbit (10 nm gold conjugate) antibody and enhanced with nucleated silver. Scale bar, 100 μm.

haemolymph and plunged into liquid-nitrogen-cooled propane maintained at −185 °C (KF80 Immersion Cryofixation System, Reichert-Jung, Vienna, Austria). Specimen ice was substituted for acetone at −80 °C, specimens were embedded in Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, West Germany) and polymerized by indirect ultraviolet illumination at −40 °C.

Polymerized blocks were sectioned on an Ultracut-E microtome (Reichert-Jung) with glass knives to a thickness of 0.5 μm and mounted on poly-*L*-lysine-coated microscope slides.

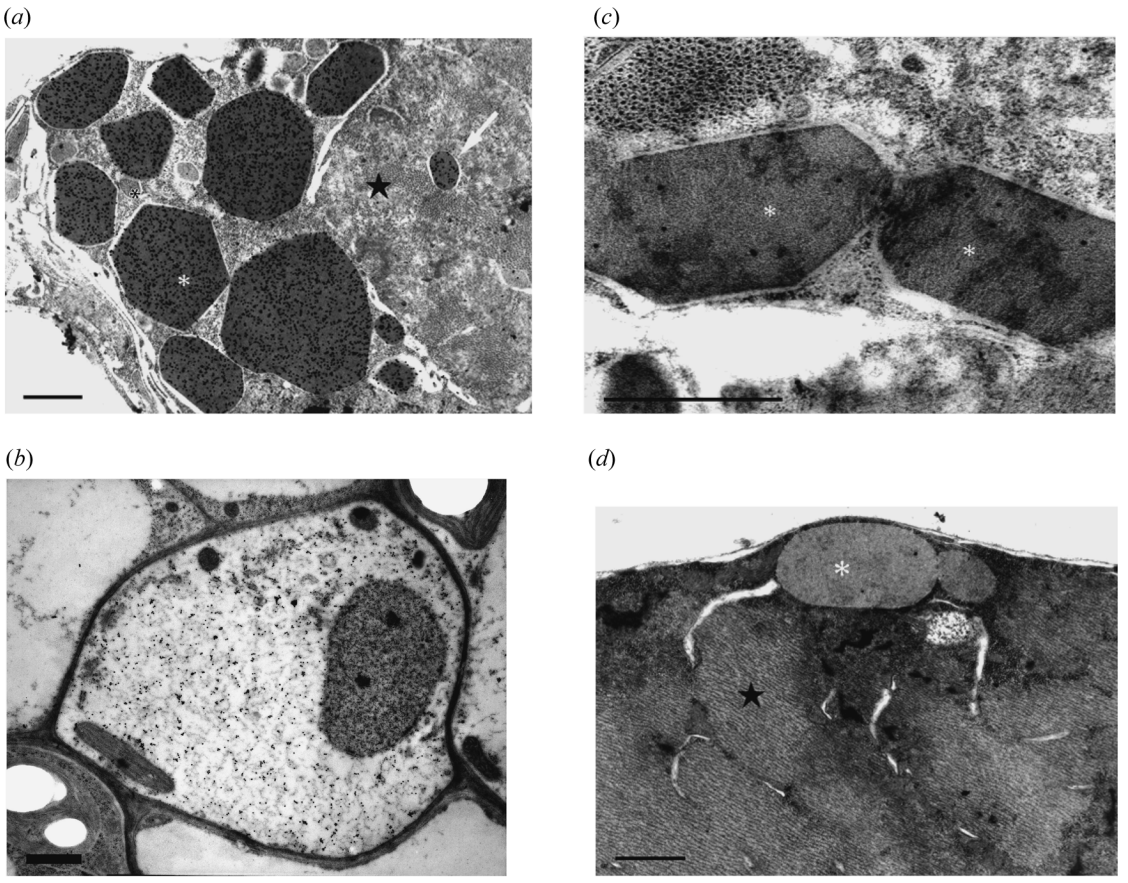

Figure 3. TEM of immunolabelled myrosinase on microbodies contained within a peripheral sheath of thorax muscle of *Lipaphis erysimi*, and a plant phloem parenchyma myrosinase cell. (*a*) Crystalline microbodies densely stained with anti-aphid myrosinase antibody. A microbody is also visible within the muscle fibres (white arrow). (*b*) Transmission electron micrograph of immunolabelled myrosinase on a transverse section through the proximal region of the leaf petiole of *Brassica juncea*. At high magnification this shows that labelling is restricted to the myrosin parenchyma cells (anti-plant myrosinase antibody, K089). (*c*) An aphid control section incubated with preimmune serum shows crystalline microbodies. (*d*) Conventionally fixed, osmicated tissue shows microbodies within a peripheral sheath of the muscle. Sections (*a*)–(*c*), after treatment with primary antibody, were stained with goat anti-rabbit antibody (20 nm colloidal gold). Black star, muscle tissue; black asterisks, mitochondria; white asterisks, dense microbodies. Scale bars, 1 μm.

(**d**) *Immunostaining*

All antibody solutions were prepared in 50 mM phosphate-buffered saline pH 7.2, containing 0.005% Tween 20 (v/v) and 0.2% cold-water fish gelatin (PBS–GT).

After blocking in 5% normal goat serum (v/v) for 1 h at 37 °C, slide-mounted sections were covered in a 20 μl droplet of primary antibody (K089, Bones & Thangstad 1991) for plant material, primary antibody dilution 1 : 1000; for aphid tissues, anti-aphid myrosinase antibody (Jones *et al.* 2001) was diluted to 1 : 1000 and incubated at 37 °C for 1 h. After three washes in PBS–GT, sections were incubated in goat anti-rabbit IgG, 10 nm diluted to 1 : 200 for 1 h at 37 °C. Sections were washed in deionized water before incubation with silver enhancement solution (Amersham, UK). Enhancement was monitored visually under a binocular microscope and halted by repeated rinsing in deionized water. Sections were counterstained in 0.1% toluidine blue, warm-air dried and mounted in Histomount (RA Lamb, Laboratory Supplies, London, UK).

(**e**) *Electron microscopy: plant and aphid tissue*

Aldehyde-fixed tissue was processed as for light microscopy and sectioned to a thickness of 90 nm on an Ultracut-E microtome with a diamond knife. Sections were collected on 400 gauge hexagonal-mesh nickel grids and processed through primary antibody solutions as specified above, then with goat anti-rabbit IgG 20 nm gold. Sections were post-stained with uranyl acetate and lead citrate, dried and viewed on a transmission electron microscope (Hitachi-7000, Hitachi, Tokyo, Japan). Controls to ensure antibody specificity were the use of rabbit pre-immune serum and the omission of primary antibody.

(**f**) *Glucosinolate analysis*

Glucosinolates were extracted from aphids in 75% methanol and, after desulphation, analysed following the method developed by Heaney *et al.* (1986).

3. RESULTS AND DISCUSSION

To establish the presence of glucosinolates in the aphid, analysis was carried out on *B. brassicae* and the non-cruciferous specialist *M. persicae*. Both aphids were fed on the mustard plant *Brassica nigra*, which contains the glucosinolate sinigrin, and were analysed 1 week later. The concentration of sinigrin per aphid for duplicate determinations was 102 and 148 ng sinigrin for *B. brassicae* and 13.2 and 12.2 ng sinigrin for *M. persicae*. These data indicate that *B. brassicae* has a mechanism for sequestering glucosinolates; in contrast, *M. persicae* accumulates very

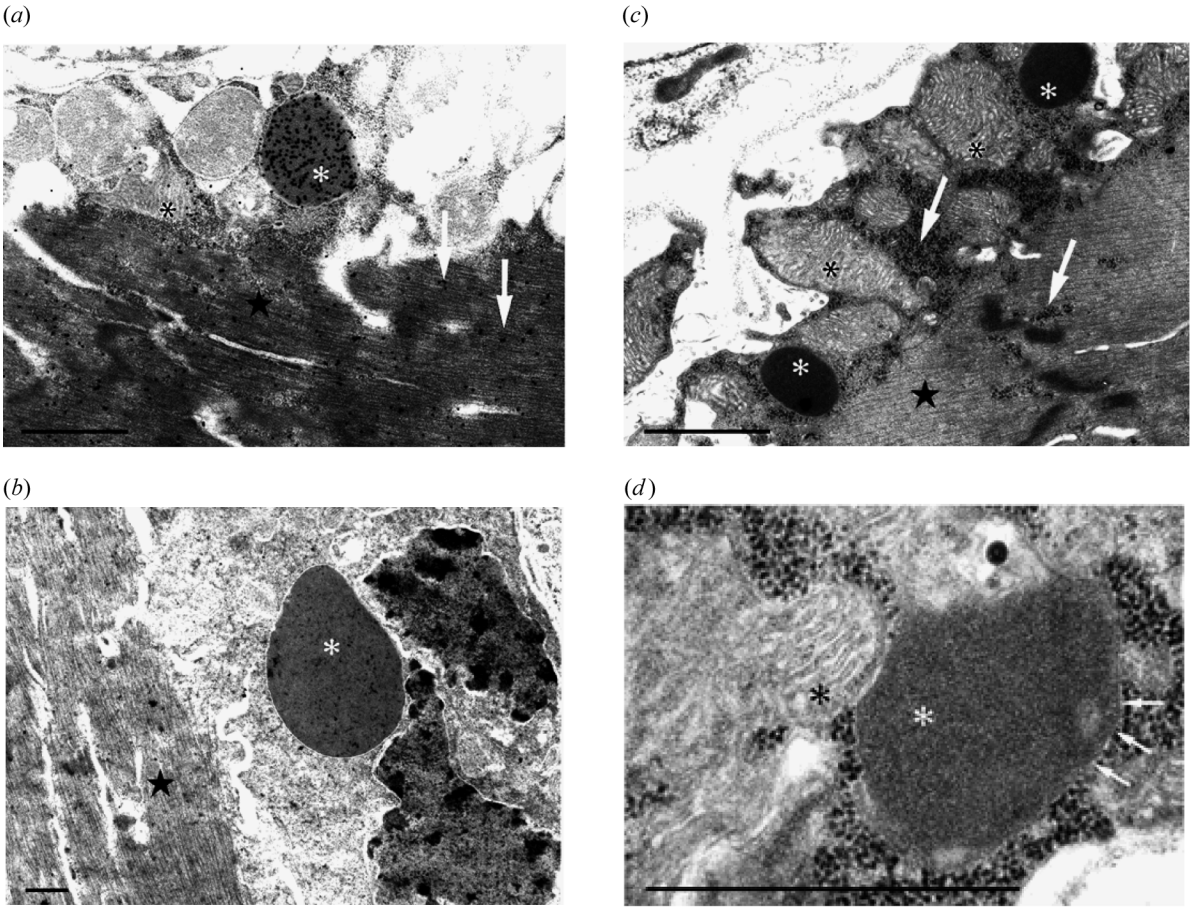

Figure 4. TEM of immunolabelled myrosinase on the thorax muscle of *Brevicoryne brassicae*. (*a*) Thorax muscle stained by immunogold labelling, showing myrosinase present in a dense microbody within the sarcoplasm of the muscle and on muscle fibres (white arrows). (*b*) Control section in preimmune serum. (*c*) Conventionally fixed, osmicated tissue showing dense microbodies in a peripheral sheath of muscle. Mitochondria and dense glycogen deposits (white arrows) are also visible. (*d*) Increased magnification of osmicated microbody shows a double membrane bounding the microbody. Excluding controls, all sections were stained with anti-aphid myrosinase antibody, followed by goat anti-rabbit antibody (20 nm colloidal gold). Black star, muscle tissue; black asterisks, mitochondria; white asterisks, dense microbodies. Scale bars, 1 μm.

little glucosinolate, which is instead found in the excreted honeydew (Weber *et al.* 1986). This is perhaps not surprising, as *M. persicae* does not contain myrosinase and there is no obvious advantage in sequestering glucosinolates. The actual location of glucosinolates in the aphid is not known at this stage, although circulation in the haemolymph is a distinct possibility, in much the same way as cyanogenic glycosides circulate in the larvae of *Zygaena trifolii* (Nahrstedt 1992).

To explore the localization of myrosinase in the aphid, monospecific antibodies (rabbit) were raised to the protein (Jones *et al.* 2001) and immunolocalization was carried out using both light and transmission electron microscopy (TEM). Light microscopy of a transverse section, taken through the abdomen of *B. brassicae* and following immunostaining, showed myrosinase to be present in muscle tissue (figure 2*b*), whereas the control gave no labelling (not shown). A longitudinal section of the head, thorax and abdomen of *B. brassicae* tissue fixed with glutaraldehyde was visualized by light microscopy using silver-enhanced immunogold localization (figure 2*d*). Silver precipitate is clearly visible over the skeletal muscle of the head, thorax and abdomen, whereas a control shows no labelling (not shown). Labelling is restricted to skeletal muscle, whereas in all sections examined flight muscles remain free of myrosinase.

Greater magnification (TEM) of the sections showed myrosinase to be present as distinct microbodies in both *L. erysimi* (figure 3) and *B. brassicae* (figure 4). In *L. erysimi* (figure 3), the microbodies were present as crystalline-like structures (figure 3*a*,*c*: hexagonal-like structures). These microbodies were intensely labelled and were within the sarcoplasm surrounding the muscles of the thorax and head, but were absent in the abdomen and in nymphs (not shown). Occasionally a microbody was observed within the muscle as an inclusion. Conventionally fixed, osmicated tissue shows dense microbodies (figure 3*d*), which appear to be globoidal in comparison to the more regular hexagonal structures present in tissue embedded in LR white and post-stained with uranyl acetate and lead citrate. Thus, it is probable that the harsh process of osmication results in a loss of definition of the microbodies. A double membrane is apparent in both osmicated and antibody-stained sections. For comparison, a magnified (TEM) phloem parenchyma cell containing myrosinase is shown (figure 3*b*). As with *B. brassicae,* myrosinase was not present in the flight muscles of *L. erysimi*. The structure of the myrosinase-containing

microbodies in *B. brassicae* (figure 4) were, in comparison to *L. erysimi* (figure 3), less well defined, although there was some indication (figure 4*a*) that hexagonal-like structures exist. It is possible that, in the fixation process, the integrity of the crystalline microbodies is affected, resulting in some labelling of the surrounding muscle tissue. A control section is shown in figure 4*b* and at a higher magnification in figure 4*d*, whereas an osmicated section is shown in figure 4*b*.

Clearly the myrosinase–glucosinolate system in the two specialist feeders is important in the life cycle of the insect. It is most probable that tissue damage to the aphid by a predator would result in the integrity of the crystalline microbodies being lost, resulting in the formation of a substrate–enzyme complex and subsequent hydrolysis, which leads to the release of volatile material. This spatial organization of the aphid myrosinase held in the muscle sarcoplasm, separate from glucosinolates that may circulate in the haemolymph, is perhaps not so different from the organization in the plant where myrosinase is restricted to myrosin cells and glucosinolates may be held separately in the 'S' cells described by Koroleva *et al*. (2000). However, in the insect, a more complex regulated mechanism of myrosinase–glucosinolate interaction seems probable, particularly as isothiocyanate release may possibly occur when injury or death is not a prerequisite for communication. In aggregated insects, especially in groups such as aphids, kin selection could be a dominant evolutionary mechanism. It is probable that the myrosinase–glucosinolate system in the specialist cruciferous aphid feeders is most important in the early stages of plant colonization, when a warning signal for predator attack is at its most useful. It is known that isothiocyanates are toxic to a range of plant fungi and insects (Chew 1988; Bones & Rossiter 1995). Thus, a possible direct defence role, in terms of the generation of toxic metabolites for the myrosinase–glucosinolate system against entomopathogenic fungi, and the parasitic wasp *Diaeretiella rapae,* cannot be discounted. As with specialist crucifer-feeding insects of plants (see, for example, Bartlet *et al*. 1999), these specialist pathogens and predators are probably adapted to the toxic glucosinolate breakdown products, but their effect on non-specialists remains to be determined.

Other insects that use glucosides are the Lepidoptera, in which it has been shown that β-*O*-glucosidase and hydroxynitrile lyase activity, and cyanogenic glucosides, are found together in the haemolymph, integument and organs of the larvae of *Z. trifolii*, in varying amounts (Nahrstedt 1992). Here, the mechanism of compartmentalization of enzyme and substrate in the haemolymph is an open question, although some authors have suggested that pH optima of linamarase may be a factor in determining regulation of activity. In addition, both calcium and magnesium ions appear to inhibit linamarase in the haemolymph of the insect (Nahrstedt & Mueller 1993). Insects have evolved other methods to avoid the toxicity of sequestered secondary metabolites, such as that of the neotropical butterfly, *Heliconius sara,* which can avoid the harmful effects of the cyanogenic leaves of *Passiflora auriculata* by metabolizing the nitrile group of the cyclopentene ring to a thiol (Engler *et al*. 2000).

Clearly, the way in which the myrosinase–glucosinolate system operates in *B. brassicae* and *L. erysimi* is very different from the cyanogenic–glucoside system in other insects and, at present, its organization appears to be unique in the insect world, but draws a direct parallel with the plant system.

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