Chapter 4
Glucosinolate-Myrosinase System and Its Role in Specialist and Generalist Insect Herbivores

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4.1 Introduction

In nature, all plants are armed with some type of protection mechanisms against pest attacks. Those can be biophysical or biochemical adaptations. Biophysical defense includes cuticular waxes, prickles, and thorns, while the latter mechanisms typically contain the synthesis of low molecular weight natural compounds, referred to as secondary metabolites, which might be unfavorable to the organisms attacking plants. Chemical defense compounds may be constitutively present in the plant, i.e., they preexist in anticipation of an insect attack (phytoanticipins), or their biosynthesis may be inducible (phytoalexins) (VanEtten et al. 1994; Mithöfer and Boland 2012). These compounds are stored in inactive form in plants and get activated upon herbivore damage. A number of constitutive glucosinolates are stored as non-active and relatively nontoxic compounds within the plant and are spatially separated from myrosinase. Tissue damage brings them together leading to production of more toxic compounds. This system of glucosinolates and hydrolytic myrosinases is referred to as glucosinolate-myrosinase system. The glucosinolate-myrosinase system is well studied because of the agriculturally important glucosinolate-containing crucifers (Brassicaceae), in addition to the long history of *Arabidopsis thaliana* (thale cress) as a model research organism. It is a major angiosperm family that consists of almost 375 genera and 3200 species (LeCoz and Ducombs 2006). Members of this family offer predominant sources of oilseeds, vegetables, and condiments. The damaged tissue of the *Brassica* plant releases glucosinolates (GLS), which might be then hydrolyzed by myrosinase to toxic isothiocyanates (Halkier

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and Gershenzon 2006). A sudden release of these insecticidal compounds is termed as "mustard oil bomb" (Hopkins et al. 2009). Those compounds affect insect pests of Brassicaceae both by way of antibiosis (direct toxicity) and antixenosis (insects show non-preference to the vegetation) (Hopkins et al. 2009).

4.2 Glucosinolate-Myrosinase System

Glucosinolates are a group of sulfur- and nitrogen-containing glycosides observed exclusively in the order Capparales (Fahey et al. 2001; Halkier and Gershenzon 2006). Till the mid-2018, the number of glucosinolates known from plants, satisfactorily characterized by modern spectroscopic methods (nuclear magnetic resonance spectroscopy and *mass spectrometry*), is 88. In addition, a group of partially characterized structures with highly variable evidence counts for approximately a further 49. Thus, it means the total number of characterized glucosinolates from plants is somewhere between 88 and 137 (Blažević et al. 2020) with many of them being species-specific (Agerbirk and Olsen 2012). Glucosinolate polymorphism is a totally common phenomenon among the various plant species containing such compounds (Kim et al. 2017; Mithen et al. 2010). Glucosinolates (β-thioglucoside-*N*-hydroxysulfates) consist of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain (Fenwick et al. 1983) which makes them nonvolatile and hydrophilic. Depending on their precursor amino acids and the types of modification to the side chain (R group), glucosinolates are divided into three main groups: aliphatic, aromatic or benzenic, and indole. Compounds derived from alanine, leucine, isoleucine, methionine, or valine are referred to as aliphatic glucosinolates, the ones derived from phenylalanine or tyrosine are known as aromatic glucosinolates, and those derived from tryptophan are referred to as indole glucosinolates. The R groups of maximum glucosinolates are modified from those precursor amino acids (Fahey et al. 2001). Methionine-derived glucosinolates have been stated as the most significant class of glucosinolates in *Brassica* vegetables, even though other glucosinolates from three special classes have also been detected in the edible parts of *Brassica* types (Mithen et al. 2003; Cartea and Velasco 2008).

Glucosinolate synthesis is a three-step process involving amino acid chain elongation followed by synthesis of glucon from the amino acid and chain amendment (glucon addition). Many glucosinolates are biosynthesized through sizable adjustments in the aglycone side chains involving a range of chemical modifications which include elongation, hydroxylation, o-methylation, and desaturation, in addition to glycosylation, oxidation, and acylation (Sønderby et al. 2010). Synthesis of glucosinolates happens in cytoplasm of plants followed by storage in vacuoles of various kinds of cells (Mithen 2001). The concentration of glucosinolates varies extensively depending upon species, plant parts, and agronomic and climatic situations (Font et al. 2005; Tripathi and Mishra 2007). A drastic decline in the glucosinolate concentration (specifically aliphatic ones) occurs in *B. napus* seeds during the primary 7 days of imbibition, while *de novo* synthesis of indole glucosinolates

and an aromatic glucosinolate (gluconasturtin) takes place concomitantly. Gluconasturtin is not initially present in the seed. During the following growth period, a few more glucosinolates are additionally synthesized (Clossais-Besnard and Larher 1991). On the other hand, glucosinolates occur in low concentrations in the completely expanded leaves (Porter et al. 1991). With the start of the reproductive phase of plant, there is a reduction in the concentration of glucosinolates in vegetative plant parts as well as in inflorescence, which otherwise has fairly large amounts of these compounds. In contrast to this, at some stage during seed maturation, glucosinolate synthesis takes place in siliques which are then transported to the seeds via pod shells (Rask et al. 2000). The levels of glucosinolates can also be influenced by environmental situations. An increase in the concentration of glucosinolates takes place in *Brassica* plants under drought conditions (Bouchereau et al. 1996; Jensen et al. 1996). But there is no consistent relationship between glucosinolate concentration and water stress since elevated levels of glucosinolates are also found in plants grown under moist conditions in comparison to the ones grown in dry soil (Louda and Mole 1991).

In plant tissue, myrosinase and glucosinolates are stored in separate cellular compartments wherein these are inactive as a result preventing self-toxicity (Jones and Vogt 2001). Myrosinase is a homodimer consisting of subunits with a $(\beta/\alpha)_8$-barrel structure containing eight α-helixes and β-sheets. The structure is stabilized through Zn^{2+} ion incorporated into the center of the dimer and is heavily glycosylated (Burmeister et al. 1997). The enzyme is present in the myrosin cells, observed for the first time in 1884 by Heinricher, who indicated the presence of cells differing in morphology and size in comparison to neighboring cells and suggested that they comprise myrosinase and accordingly named them as myrosin cells. The distribution of myrosin cells differs in individual plant parts as well as plant development stage. High myrosinase activity in the upper parts of roots was reported in mature rape plants, while it was lowest in stems and inflorescences (Andréasson et al. 2001).

As mentioned earlier, myrosinase and glucosinolates are stored in separate cellular compartments. Tissue damage by external factors, e.g., after pest attack or on cutting or grinding, brings myrosinase into close contact with glucosinolates leading to hydrolysis of thioglucosidi bond in glucosinolate structure. This results in cleavage of D-glucose and release of an unstable aglucon-thiohydroximate-*O*-sulfate. Depending on the parent glucosinolate, hydrolysis conditions (pH, temperature), presence of cofactors (e.g., Fe^{2+}), and additional protein elements (e.g., epithiospecifier protein (ESP) and thiocyanate-forming protein (TFP)), the aglucone undergoes rearrangements to form distinct classes of degradation products: isothiocyanates (ITC), thiocyanates, nitriles, epithionitriles (EPT), and oxazolidine-2-thiones (Fig. 4.1) (Rungapamestry et al. 2006).

The formation of unstable intermediate aglucon-thiohydroximate-*O*-sulfate results in the first step of glucosinolate degradation catalyzed by myrosinase. Isothiocyanates are formed by spontaneous rearrangement from the unstable aglucone at neutral pH (6–7). Isothiocyanate production is higher in neutral pH condition than in acidic and alkaline. For example, hydrolysis of gluconapin and sinigrin produces isothiocyanate, namely, 3-butenyl isothiocyanate and 2-propenyl

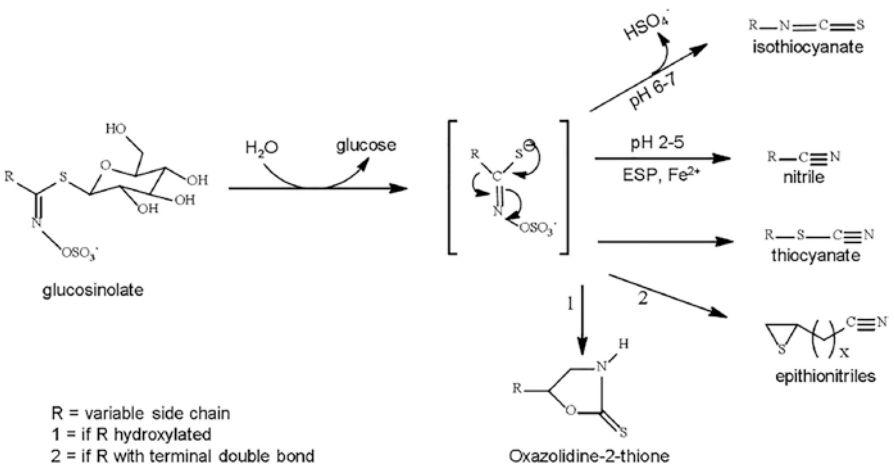

Fig. 4.1 Hydrolysis product of glucosinolates (adapted from Hennig 2013)

isothiocyanate, respectively. Isothiocyanates from indolic group are not much stable than aliphatic and benzenic group, which undergo further modifications. Interestingly, glucosinolates from the indolic group can be broken down independent of myrosinase activation in the physiological condition of the aphid gut to produce nitriles, alcohols, and unstable isothiocyanates that are further metabolized (Agerbirk et al. 2009). Generally, isothiocyanates are lipophilic, volatile, and more toxic than other hydrolysis products of glucosinolates.

At lower pH (<6), epithionitriles and nitriles are formed from thiohydroximate-*O*-sulfonate with the help of epithiospecifier protein. In the presence of Fe^{2+} ions and myrosinase, the recombinant protein catalyzed the formation of epithionitriles from thiohydroximates derived from alkenyl glucosinolates containing double bond between carbon atoms in the side chain (de Torres et al. 2005). Most likely, Fe^{2+} ions enabled the formation of transient bond between thiohydroximate and epithiospecifier protein because this reaction takes place only for thiohydroximate-*O*-sulfonate intermediate and not native glucosinolates (Foo et al. 2000; de Torres et al. 2005). The activity of epithiospecifier protein can be variable in individual plant parts and the development stage. A study on cuckooflower (*Crambe abyssinica*) sprouts suggests that the highest activity of epithiospecifier protein takes place during the second day of sprouting with gradual drop over 3 consecutive days and till the fifth day, when the activity becomes stable at the low level. The changes in the myrosinase activity are similar in trend; but the fluctuations are not as huge as in the case of epithiospecifier protein (Williams et al. 2009). The measurements of epithionitriles liberated in *Brassica* vegetables also revealed the great variability among different plant parts. Interestingly, crushed seeds and sprouts contained higher quantities of these compounds than aerial parts, including edible parts of the same plant (Kołodziejski et al. 2019).

When the glucosinolates are nonalkenyl, nitriles are formed by epithiospecifier protein from the intermediate hydrolysis product of glucosinolates. Apart from epithiospecifier protein, the presence of nitrile-specifier protein in plants also promotes the nitrile production which has different amino acid sequence than that of epithiospecifier protein (Wittstock and Halkier 2002). Nitrile-specifier proteins have been identified in rutabaga (*Brassica napus* L. var. *napobrassica*) (Wittstock and Halkier 2002) and thale cress (*Arabidopsis thaliana*) (Wittstock et al. 2016). Similar to epithiospecifier protein, nitrile-specifier protein also varies with stage and parts of the plants (Wittstock et al. 2016). Interestingly, nitrile-specifier protein has also been reported in large white butterfly, *Pieris brassicae*, that feeds exclusively on *Brassica* plants. *P. brassicae*, being *Brassica* specialist, has evolved mechanism to redirect the degradation of glucosinolates to produce less toxic nitriles with the help of nitrile-specifier protein from otherwise more toxic isothiocyanates produced during myrosinase-catalyzed hydrolysis.

Recently, epithiospecifier modifier protein was recognized in thale cress (*Arabidopsis thaliana*), a myrosinase-associated protein that modifies the activity of other specifier proteins operating in the same plant to promote the formation of isothiocyanates. It was shown to negatively affect the nitrile-to-isothiocyanate ratio in glucosinolate degradation. Even though the mechanism is not much clear, one of the feasible mechanisms is direct interplay of epithiospecifier modifier protein with myrosinase to stimulate the greater production of isothiocyanates and/or with epithiospecifier protein to suppress their activity responsible for epithionitriles and nitrile formation. It has been suspected as an evolutionary mechanism to counter the detoxification mechanism of isothiocyanates by insect herbivores such as the presence of nitrile-specifier protein in *P. brassicae* that redirects the degradation of glucosinolates from production of isothiocyanates to nitriles. However, the activity of epithiospecifier modifier protein on nitrile-specifier protein remains to be examined (Zhang et al. 2006).

Thiocyanates, the hydrolysis products of glucosinolates, are formed after enzymatic degradation of glucosinolates catalyzed by myrosinase with the help of thiocyanate-forming protein. Thiocyanate-forming proteins have been reported in garden cress (*Lepidium sativum*) (Burow et al. 2007), thale cress (*Arabidopsis thaliana*), and broccoli (*Brassica oleracea* L. var. *italica* Plenck) (Burow et al. 2006, 2007; Morant et al. 2008; Williams et al. 2008). The synthesis of thiocyanates occurs very occasionally in plant tissues as compared to isothiocyanates, nitriles, and epithionitriles. Further, only a handful of glucosinolates (such as glucotropaeolin, sinigrin, and glucoerucin) can function as precursors for the formation of suitable thiohydroximes because their chemical structures permit the formation of a strong carbocation form necessary for the reactions leading to thiocyanates (Kuchernig et al. 2011). Depending on the side-chain structure, thiocyanate-forming protein catalyzes the aglucone conversion into either thiocyanates or epithionitriles or nitriles (Kuchernig et al. 2011). Further, environmental conditions such as pH or availability of ions also determine the final product to be formed.

4.3 Impact of Glucosinolate Hydrolysis Products on Specialist and Generalist Insect Pests of Brassicaceae

Insect pests may be either generalist or specialist herbivores. Those which feed on a range of hosts belonging to different families are considered as generalist herbivores. They have greater resource availability and higher capability to exploit new hosts. The possibility of host-plant switching may additionally enhance insect development by minimizing the exposure to any single toxic phytochemical as well as by optimizing the nutrient intake (Bernays and Minkenberg 1997; Behmer 2009). On the other hand, specialist insect pests have a restrained host range which reduces the interspecific competition. They have the ability to counter the single toxic compound in the host plant more efficiently than generalist pest. *Brassica* plants are infested by many generalist or specialist insect herbivores from diverse insect orders. These include *Lipaphis erysimi*, *Brevicoryne brassicae*, *Pieris brassicae*, *P. rapae* and *P. napi*, *Plutella xylostella*, *Athalia lugens proxima*, *Trichoplusia ni*, *Mamestra brassicae*, *Chromatomyia horticola*, *Delia radicum*, *Phyllotreta cruciferae*, *Dasineura brassicae*, *Spodoptera exigua*, *S. littoralis*, *S. gregaria*, and *Helicoverpa armigera* (Agrawal 2000; Traw and Dawson 2002; van Poecke et al. 2003; Reymond et al. 2004; Mewis et al. 2006; Kuśnierczyk et al. 2007; Vogel et al. 2007; Travers-Martin and Müller 2008; Poelman et al. 2008; Bidart-Bouzat and Kliebenstein 2011).

Brassicaceae have evolved glucosinolate-myrosinase system to fend off any insect attack. Among the hydrolysis products of glucosinolates, isothiocyanates are the most reactive and functional metabolites toxic to insects than other hydrolysis products (El Sayed et al. 1996; Borek et al. 1998). Mechanism of their toxicity involves disruption of amino groups of proteins. The lipophilic properties of isothiocyanates facilitate passive diffusion into the insect cell through cell membrane, where they react with proteins to cleave the disulfide bond resulting in impaired catalytic activity (Kawakishi and Kaneko 1985, 1987; Halkier and Gershenzon 2006).

The myrosinase glucosinolate system is a double-edged sword. On one side, it protects the plants from generalist feeders (Rask et al. 2000), while on the other side glucosinolates make plants more attractive to specialist feeders (Renwick 2002; Bjorkman et al. 2011). Further, toxicity of glucosinolate varies depending upon the class (aliphatic, indolic, and benzenic glucosinolates) and the insect involved. Generalist insect pests tobacco hornworm, *Manduca sexta*, and the cabbage looper, *Trichoplusia ni*, are negatively affected only by the presence of aliphatic glucosinolates (Müller et al. 2010), while green peach aphid, *Myzus persicae*, is affected by indolic glucosinolates (de Vos and Jander 2009; Pfalz et al. 2009). In contrast, beet armyworm, *Spodoptera exigua*, is adversely affected by the presence of both aliphatic and indolic glucosinolates (Müller et al. 2010). Further, glucosinolates exhibit exclusive effects depending on the herbivore. For instance, the presence of higher concentrations of sinalbin in cotyledons of *Sinapis alba* exhibits antibiotic resistance to bertha armyworm, *Mamestra configurata*, in terms of low survival and low body weights and repellent effect to the flea beetle, *Phyllotreta cruciferae*. The

lower concentrations found in older leaves did no longer seem to provide any protection against either species. Alternatively, specialist insect herbivores have adapted to use glucosinolates to their advantage. Glucosinolates are known to act as oviposition and feeding stimulants for more than 25 insect species of the orders Coleoptera, Lepidoptera, and Diptera (Hopkins et al. 2009). Many insects such as cabbage aphid, *Brevicoryne brassicae* (Nottingham et al. 1991), and diamondback moth, *Plutella xylostella* (Renwick et al. 2006), carry receptor neurons that could detect isothiocyanates. They are known to stimulate oviposition in large white butterfly, *Pieris brassicae*; small white butterfly, *Pieris rapae* (Renwick et al. 1992; Smallegange et al. 2007); diamondback moth, *Plutella xylostella* (Renwick et al. 2006); and cabbage fly, *Delia radicum* (Roessingh et al. 1992). Higher allyl glucosinolates (sinigrin) induce feeding in cabbage aphid, *Brevicoryne brassicae* (Lankau 2007). Some other plant constituents also act as feeding stimulants collectively along with glucosinolates. Flavonoids and glucosinolates increase the feeding of flea beetle, *Phyllotreta armoraciae* (Nielsen et al. 1979), and diamondback moth, *Plutella xylostella* (van Loon et al. 2002). A number of other hydrolysis products are also known to affect insect feeding/behavior either directly or indirectly. Phenylacetonitrile from benzenic glucosinolate breakdown acts as an indirect plant defense in two different ways, one by repressing the mating of female pierid butterflies (anti-aphrodisiac effect) and another by attracting natural enemies such as the generalist egg parasitoid, *Trichogramma brassicae* (Hymenoptera) (Andersson et al. 2003; Fatouros et al. 2008).

Since glucosinolates play a defensive role in plants, it raises the question that double zero ("00") canola plants which are low in these compounds might be vulnerable to many insects. Such questions may be misplaced because low glucosinolate levels in "00" canola plants were confined primarily to seeds (Milford et al. 1989) and high and low glucosinolate cultivars did not differ in their susceptibility to pod midge (*Dasineura brassicae*), though the level of glucosinolates in green tissue was not determined (Åhman 1982). Extensive studies in India with both *B. juncea* and *B. napus* have shown no reasons to believe that canola-quality cultivars were more susceptible than their non-canola counterparts (Kumar 2019). Theoretically (though there are no supporting references), low glucosinolate plants may be less attractive to specialist insects for which these compounds serve as feeding and oviposition stimulants (Gabrys and Tjallingii 2002; Mewis et al. 2002). This is again supported by the work of Giamoustaris and Mithen (1995) who reported that increase in content of glucosinolates in *B. napus* resulted in increased feeding damage by specialist insects, flea beetles (*Psylliodes chrysocephala*), and greater incidence of small white butterfly (*P. rapae*), while the damage by generalist pests, i.e., pigeons and slugs, was reduced. Further, glucosinolate-rich flower tissues are preferred more by *Pieris brassicae* and sustained higher growth compared to leaf tissues (Smallegange et al. 2007) indicating the selective role of glucosinolate to elicit feeding in this specialist insect and the adaptation of the insect to use these compounds to its advantage.

4.4 Insect Herbivore Adaptation of Glucosinolate-Myrosinase System of Brassicaceae

As discussed above, insects are strongly affected by glucosinolate hydrolysis products. However, a few insect herbivores have adapted to neutralize/detoxify these toxic compounds which permit them to feed on glucosinolate-containing plants. These adaptive processes play their role before and after consumption of food. Insects may either keep away from toxic compounds or feed on glucosinolate-containing plant tissues. Exposure to toxins could be often related to accelerated activity of phase I and phase II detoxification mechanisms. By oxidation, hydrolysis, or reduction, phase I enzymes introduce reactive and polar groups into their substrates. The P450 monooxygenases (P450s), which are commonly known for their role in the metabolism of natural and synthetic insect pesticides, are prominent among phase I enzymes. Following phase I, the activated metabolites of xenobiotics are conjugated with compounds which include glutathione (GSH), sulfate, or glucuronate in phase II reactions. The glutathione-*S*-transferases (GST) are among the best recognized of the phase II enzymes, and increases in their levels are related to resistance to toxins. Some insects can also sequester the toxins in their body to protect them from natural enemies. In insects, behavioral, physiological, and metabolic adaptations may be mixed to conquer the toxic compound.

4.4.1 Before Consumption of Food: Host Plant Selection and Feeding Guilds

Insects require resources for growth and reproduction. Besides the quantity of dietary consumption, the quality of the food consumed can be critical for development time, fecundity, and fitness (Awmack and Leather 2002). The usage of an extensive variety of hosts increases food availability and allows mixtures of different kinds of food, which might also improve nutrient stability (Simpson and Raubenheimer 2001; Berner et al. 2005). Dietary mixing also helps to dilute probably toxic allelochemicals which might be unevenly distributed over different plants (Freeland and Janzen 1974; Bernays and Minkenberg 1997) or even in the same plant, i.e., varying concentrations in different organs and developmental stages (Hoy et al. 1998; Gebrehiwot and Beuselinck 2001), or are induced by the feeding herbivore itself (van Dam et al. 2000). Generalists are known to feed on a wide range of plant species, often from more than one plant family. They are adapted to low to medium levels of various plant defense compounds to avoid ingestion of any single lethal doses of phytotoxin. For example, generalist grasshopper species record higher growth, survival, and fecundity through host plant switching and dietary mixing, while feeding is restricted to a single plant species (Bernays and Minkenberg 1997).

Both generalist and specialist insects do host plant switching and dietary mixing either within the same plant or among the populations of plants or between two plants from different families. Whereas generalists benefit from suppressing any degree of toxicity from plant defense compounds, permitting at least short-term feeding, specialists often suppress only high levels of toxicity and benefit from the presence of low to intermediate levels of plant defense compounds. Sequestering specialists selectively take up and store chemical plant compounds in their own body, benefitting from sequestered compounds because sequestered compounds shield the insect from their enemies (Sect. 4.4.2.5) (Nishida 2002; Ali and Agrawal 2012). Behavioral adaptation via host-plant switching in generalists and selection of toxic plants in specialists also involve trade-offs and fitness costs. Insect herbivores need to invest time and energy to search for an appropriate host (Schultz 1983; Dethier 1988; Despres et al. 2007). Investment costs differ in generalists and specialists and especially seem to depend not only on the level of plant defense compound but also on an excessive degree of the level of activating enzyme. Frequently the more generalists want to interchange host plants, the longer they need to look for suitable host plants, which increases costs. In contrast, specialists need to invest much less time, energy, and thus costs in this case.

Insect herbivores can acquire suitable food through specialist and generalist feeding habits (Schoonhoven et al. 1998). Depending upon the mouthparts, agricultural insect pests are typically divided into two groups: chewing insects and piecing and sucking insects. Those with chewing mouthparts crush the leaf/plant tissue with the help of mandibles, maxillae, labrum, and labium. Examples of chewing insects include grasshoppers (order Orthoptera), beetles (order Coleoptera), and larval Lepidoptera. On the other hand, in sucking insects (order Hemiptera), mandibles and maxillae are modified into a long proboscis protected by a modified labium which penetrates the plant tissue to feed on the plant sap.

Tissue damage during feeding brings together glucosinolates and myrosinases which otherwise are spatially separated in vacuoles of cells and myrosin cells, respectively. Tissue damage depends upon the feeding guild and herbivore species (Textor and Gershenzon 2009).

A guild is defined as a group of species similarly exploiting the same class of environmental resources. In general, there are distinctive guilds of feeding including leaf-chewing, leaf-mining and piercing-sucking (Bernays and Janzen 1988; Sinclair and Hughes 2010). Chewing insects are exposed to more toxic compounds due to higher tissue damage than other feeding guilds, but physiological conditions may favor stabilization or detoxification of plant defense compounds as discussed later. Compared to chewing insects, feeding by leaf miners is limited to parenchymal or epidermal tissues leading to production of canals, mines, or blotches (Sinclair and Hughes 2010). Thus, the overall tissue damage by leaf miners is less than tissue feeders but more than piercing and sucking insects (Schappert and Shore 1999). The limited tissue damage leads to limited production of hydrolysis products compared to chewing insects.

In contrast, sucking insects such as aphids are exposed to little or no plant defense due to their specialized feeding habit. Aphids are specialized phloem sap feeders

which insert their needle-like stylets in the plant tissue keeping off/counteracting the different plant defenses. They withdraw large quantities of phloem sap while maintaining the phloem cells alive. In comparison to the insects with biting and chewing mouthparts which tear the host tissues, aphids penetrate their stylets between epidermal and parenchymal cells to finally reach sieve tubes with slight physical harm to the plants, which is rarely perceived by the host plant (Bhatia et al. 2011). The long and flexible styles travel within the apoplasm across intercellular areas (Giordanengo et al. 2010), while styles also perform intracellular punctures to investigate a cell's internal chemistry (Züst and Agrawal 2016). The excessive stress inside sieve tubes enables in passive feeding (Bhatia et al. 2011). During the stylet penetration and feeding from phloem, aphids produce two types of saliva which are used to form sheath around the stylet (gelling saliva) and to prevent coagulation of proteins which is helpful to defend the feeding site from plant's immune response and efficient feeding (watery saliva), respectively. Aphids feed only on single phloem cells, so the myrosinase-catalyzed breakdown of glucosinolates into active hydrolysis products may not be triggered because glucosinolates and myrosinases are assumed to be placed in separate cells (Barth and Jander 2006). Some specialist insects have adapted to use glucosinolates for their own benefit. For example, cabbage aphid, *Brevicoryne brassicae* (Aphididae), sequesters and stores glucosinolates in its body which are later used in defense against predators (Bridges et al. 2002). Likewise, the generalist green peach aphid, *Myzus persicae* (Aphididae), is capable of ingesting intact glucosinolates which are later excreted out in nontoxic form in the honeydew (Barth and Jander 2006).

Induction of plant defenses also depends on the insect feeding guilds. Induced resistance is a physiological state of enhanced defensive capacity of the plant induced through biological or chemical inducers, which protects plant tissues not exposed to the initial attack against future attack by herbivores that may show greater resistance both locally and systemically (van Loon et al. 1998). While feeding, insects deposit small amounts of saliva/oral secretions at the disrupted tissue. Active components in these fluids (the so-called herbivore-associated elicitors (HAEs) or herbivore-associated molecular patterns (HAMPs)) can be perceived by a large number of plant species as chemical cues (Alborn et al. 1997, 2007; Musser et al. 2002; Schmelz et al. 2009). The perception of herbivore-associated elicitors induces precise defense responses which differ from simple mechanical damage in most of the cases (Mithöfer and Boland 2008; Bonaventure et al. 2011). The perception of herbivore-associated elicitors and herbivore-associated molecular patterns by plants usually affects the activation of particular plant responses in order to protect or tolerate an insect attack. Such reactions include, among others, unique changes in metabolism, gene expression, and plant growth and development patterns (Turlings et al. 1990; Kessler and Baldwin 2002; Bede et al. 2006). During herbivore attack, insect-associated elicitors bind to putative receptors at the plasma membrane and prompt downstream responses such as depolarization of cellular membranes and the activation of Ca^{2+} inflow. Feeding by a few insects induces very strong modifications in cellular membranes and Ca^{2+} influx (Maffei et al. 2004; Arimura et al. 2008). This Ca^{2+} inflow depends on the activity of Ca^{2+} channels; the

reaction can be reduced through either specific Ca^{2+} channel inhibitors or calcium chelators (Maffei et al. 2004, 2006). Modifications in cellular membrane potential and Ca^{2+} influx activate NADPH oxidases in cells that catalyze the production of superoxide such as reactive oxygen species (ROS) (Sagi and Fluhr 2001, 2006). These reactive oxygen species modify amino acids in regulatory proteins as a redox-based mechanism to translate secondary signals into the transcriptional activation of defense-associated genes, in particular lipoxygenases, to initiate the biosynthesis of jasmonic acid (Porta and Rocha-Sosa 2002).

Chewing insects lead to higher degradation of glucosinolates due to greater tissue damage compared to piercing-sucking insects which inflict limited tissue damage (Barth and Jander 2006; Textor and Gershenzon 2009) coupled with active manipulation of plant defenses (Miles 1999; Will et al. 2007, 2009, 2013).

4.4.2 After Consumption of Food

4.4.2.1 pH of Insect Gut

The pH of the insect midgut lumen ranges from a highly acidic pH 3.1 to an extremely alkaline pH 12–14 among different insect orders (Berenbaum 1980; Schultz and Lechowicz 1986; Appel and Joern 1998; Harrison 2001; Cristofoletti et al. 2003). It is promoted by the active secretion of K^+ into the midgut in alternation for H^+ through the proton ATPase pump and by transport of ammonia from the gut lumen into the hemolymph in some insects which are carried out in goblet and collumner cells of the midgut (Weihrauch 2006). Vacuolar proton pumps are reported to occur in many secretory tissues (Huss et al. 2011). A proton pump occurs within the apical membrane of insect salivary glands (Baumann and Walz 2012) and in Malpighian tubule cells and drives the formation of fluid in Malpighian tubules. The pH of insect gut influences the movement of any enzymes secreted into or carried with food. In addition, gut pH might also influence the solubility of ingested components, the toxicity of some potential toxins, and the population of gut microorganisms. The classical example of detoxification of plant toxin by gut pH is tannin detoxification by herbivores. The higher midgut pH in the insects feeding on tannin-rich food may have evolved as a defense mechanism to reduce the toxicity of tannins, which have a tendency to form complex with proteins. The presence of acidic and alkaline pH in the midgut of insect pest of *Brassica*s reduces glucosinolate hydrolysis (El-Shora et al. 2016) which helps the insects to sequester the glucosinolates in their body that are used to protect them from natural enemies (Sect. 4.4.2.5). A numerous insect herbivores with an alkaline/acidic midgut are known to feed on plants not protected by toxins (Berenbaum 1980). Therefore, pH of the midgut probably did no longer rise up as an evolutionary reaction to glucosinolates of plants. Insect herbivores with an alkaline midgut absolutely may have been pre-adapted to feed on plants protected by glucosinolates.

4.4.2.2 Glutathione *S*-Transferases (GST): After Formation of Hydrolysis Product Isothiocyanates

Isothiocyanates are the glucosinolate breakdown products most often encountered by herbivores feeding on glucosinolate-containing plants. Because of their lipophilic nature, isothiocyanates are absorbed passively into epithelial cells. If they are not detoxified at this stage, they would possibly be dangerous to the cells as they easily react with amino groups of proteins and are reported to cleave disulfide bonds though in vitro (Kawakishi and Kaneko 1985, 1987). Isothiocyanates are conjugated with glutathione as quickly as they enter the cells by conjugating enzymes (Kassahun et al. 1997; Traka and Mithen 2009). Conjugating enzyme increases the conjugate water solubility and excretion efficiency. Glutathione (GSH) is an essential biological nucleophile and a reducing agent and is normally found in cells. It is a Glu-Cys-Gly tripeptide which binds with electrophilic centers of isothiocyanates through glutathione *S*-transferases. Isothiocyanate-glutathione conjugates are actively transported out of the cells where they either enter the mercapturic acid pathway for renal excretion or dissociate to release the free isothiocyanates (Al Janobi et al. 2006; Traka and Mithen 2009).

As conjugation with glutathione is a classical and ubiquitous phase II metabolism reaction, it has been investigated whether or not isothiocyanates are detoxified by conjugation with glutathione in insect herbivores. Conjugation with glutathione has been shown to involve in detoxification of isothiocyanates in many generalist herbivores with varying glucosinolate preferences (Schramm et al. 2012). Generalist insect pests such as cotton bollworm *Helicoverpa armigera*, fall armyworm *Spodoptera frugiperda*, cabbage moth *Mamestra brassicae*, and cabbage looper *Trichoplusia ni,* which feed on glucosinolate-containing plants, conjugate poisonous isothiocyanates with glutathione by glutathione-*S*-transferase activity leading to the formation of nonpoisonous products which are excreted in the frass (Schramm et al. 2012). Despite the fact that phase II metabolism response is classical and ubiquitous, glutathione-*S*-transferase activity on isothiocyanates is insect-specific. Extracts acquired from midgut tissue of velvet bean caterpillar, *Anticarsia gemmatalis*—an insect herbivore that does not feed on glucosinolate-containing plants—lacked glutathione-*S*-transferase activity toward isothiocyanates (even after induction), but not on artificial substrates frequently used for glutathione-*S*-transferase activity measurements (Wadleigh and Simon 1988; Yu 1987). This seems to signify that an alternatively specific glutathione-*S*-transferase activity in gut tissue, at least, contributes to isothiocyanate detoxification in the two glucosinolate-feeding species.

Piercing-sucking insects such as aphids, unlike chewing herbivores, cause only minor tissue damage during feeding and are thought to prevent the detonation of the so-called mustard oil bomb to a large extent. These herbivores guide their stylets among individual plant cells to the phloem sieve elements (Tjallingii and Esch 1993). Consequently, glucosinolates present in the phloem and apoplast aren't brought into contact with myrosinases which are localized in separate myrosin cells (Thangstad et al. 2004; Barth and Jander 2006). Thus, green peach aphid, *Myzus*

persicae, is not too much by the presence of glucosinolates in plant tissues. However, indolic glucosinolates breakdown under the conditions present in the insect gut independent of plant myrosinases and have a strong antifeedant effect on *M. persicae* (Kim and Jander 2007; Kim et al. 2008). *M. persicae* is known to harbor a gut-expressed gene with similarity to plant myrosinases (Ramsey et al. 2007). The breakdown products detected in the aphid honeydew contain amino acids and glutathione conjugates that represent lively detoxification products (Ramsey et al. 2010). Induction of glutathione-*S*-transferase activity in response to increasing glucosinolate concentrations has been shown in *M. persicae* (Francis et al. 2005). Interestingly, leaf miner, *Scaptomyza* species (specialist), has also been discovered to excrete glutathione conjugation products with isothiocyanates (Gloss et al. 2014). Glutathione-*S*-transferase-dependent detoxification has an excessive metabolic cost. Glutathione levels in *Spodoptera littoralis* and *Myzus persicae* midgut tissues and hemolymph have been found to drop significantly upon ingestion of isothiocyanates in a dose-dependent manner, suggesting that the available pool of glutathione-*S*-transferase is confined (Kim et al. 2008; Jeschke et al. 2016). These metabolic changes resulted in other metabolic outcomes such as decreased weight and body protein levels and reduction in fecundity, with some of these outcomes ameliorated by supplementation of cysteine (precursor of glutathione) (Jeschke et al. 2016). However, generalist herbivores can modify their feeding behavior upon encountering isothiocyanates to keep away from leaf regions of high constitutive (Shroff et al. 2008) or induced glucosinolates (Perkins et al. 2013).

4.4.2.3 Nitrile-Specifier Protein: After the Formation of Intermediate Hydrolysis Product

Cabbage butterflies, *Pieris* spp., are known to secrete a protein into the gut lumen, specified as nitrile-specifier protein (NSP), which interferes with myrosinase-catalyzed glucosinolate hydrolysis in the ingested plant tissue (Wittstock et al. 2004). This nitrile-specifier protein redirects the hydrolysis from toxic isothiocyanates to simple nitriles which are excreted with the feces, either unchanged or after further metabolism. This mechanism has been identified in numerous different pierid species specialized to feed on glucosinolate-containing plants (Wheat et al. 2007; Wittstock et al. 2004). Nitrile-specifier protein does not have hydrolytic activity on glucosinolates; rather, it acts on the glucosinolate aglucone, the product of plant myrosinase-catalyzed hydrolysis of the thioglucosidi bond. In spite of this purposeful similarity, larval nitrile-specifier protein does not have any structural similarities with plant-specifier proteins. *Arabidopsis thaliana* has AtNSP1 (At3g16400) and AtNSP2 (At2g33070), but in comparison to *Arabidopsis* epithiospecifier protein, *Pieris rapae* nitrile-specifier protein has a low substrate specificity and isn't always dependent on Fe^{2+} (Burow et al. 2006, 2009).

So far, nitrile-specifier protein has most effective been located in glucosinolate-feeding pierid species. Approximately ten million years after the evolution of the glucosinolate-myrosinase system, ancestral pierid insects evolved a key

biochemical adaptation that allowed them to make use of Brassicales plants as their food source (Wheat et al. 2007; Beilstein et al. 2010). This host shift to Brassicales plants becomes facilitated by the evolution of a nitrile-specifier protein, which directs the myrosinase-catalyzed breakdown of glucosinolates in the larval gut to form nitriles, which can be less toxic and reactive than isothiocyanates (Wittstock et al. 2004). These nitriles may be further metabolized prior to excretion. Simple nitriles derived from aliphatic glucosinolates are excreted unchanged, while the nitriles derived from benzenic glucosinolates undergo further metabolism to its sulfate ester (Müller et al. 2003; Wittstock et al. 2004). Phenylacetonitrile shaped from benzylglucosinolate could yield either *N*-phenylaceryglycine or hippuric acid/*N*-benzolylisoserine from the intermediate phenylacetic acid by nitrilase and nitrile hydratase activity, respectively. Although *Pieris* larvae evidently have an efficient method to avoid toxic isothiocyanates, the formation of cyanide ("cyanide bomb") during metabolism of benzenic glucosinolates may also result in toxicity (Stauber et al. 2012). The presence of constitutive β-cyanoalanine synthase and rhodanese in the gut detoxifies the cyanide to nonpoisonous form (van Ohlen et al. 2016). Based on metabolite analyses and the experimentally demonstrated ability of *P. rapae* to survive in cyanide fumigation experiments as well as the facts that benzylglucosinolate was one of the predominant glucosinolates in ancient Brassicales and that ancient Brassicales lack nitrilases involved in alternative pathways, Stauber et al. (2012) proposed that the ability of pierid species to safely handle cyanide contributed to the primary host shift from Fabales to Brassicales that occurred about 75 million years ago and was followed by pierid species diversification.

4.4.2.4 Glucosinolate Sulfatase: Before the Hydrolysis by Myrosinase

Another way to conquer the glucosinolate-myrosinase system could be to rapidly metabolize the intact glucosinolates earlier than they are hydrolyzed by plant myrosinases in the ingested tissue. Given the high levels of myrosinase activity in plant tissues, this will require highly efficient metabolizing enzymes or a myrosinase inhibitor to be present in the mouthparts and/or gut of the herbivore. The enzyme glucosinolate sulfatase (GSS) converts intact glucosinolates to desulfoglucosinolates which are not recognized by myrosinase (Matile 1980). Glucosinolate sulfatase activity has been reported in diamondback moth, *Plutella xylostella*; desert locust, *Schistocerca gregaria*; and turnip sawfly, *Athalia rosae* (Ratzka et al. 2002; Falk and Gershenzon 2007; Opitz et al. 2011). *P. xylostella* larvae possess a glucosinolate sulfatase in their gut that converts all primary classes of glucosinolates to desulfoglucosinolates, which are not amenable to myrosinases (Ratzka et al. 2002). The expression of glucosinolate sulfatase is under tight developmental and tissue-specific regulation: transcripts are constitutively present in the larval gut—the only stage and organ exposed to glucosinolates in the diamondback moth life cycle—and the sulfatase transcripts are absent in other tissues and developmental stages (Ratzka et al. 2002).

Desert locust, *Schistocerca gregaria* (generalist), is also known to possess a glucosinolate sulfatase in the gut with high substrate specificity. Glucosinolate sulfatase activity enables the insect to feed even on *Schouwia purpurea*, a plant with very high concentrations of glucosinolates (Falk and Gershenzon 2007). The glucosinolate sulfatase activity increased tenfold when locusts were fed on *S. purpurea*, while it was reduced when glucosinolates were eliminated from diet. This indicates that glucosinolate sulfatase activity is highly inducible in generalist desert locust, while it is constitutive in the specialist diamondback moth larvae. The interplay between specialized insect enzymes that are active before plant myrosinase and sequestration, as a further adaptation, was proven in the sawfly *Athalia rosae*. Sequestered glucosinolates are hastily turned over and leave the insect body within a day upon diet change (Müller and Wittstock 2005). Therefore, *A. rosae* larvae are required to continuously feed on glucosinolate-containing plants to hold their hemolymph glucosinolate levels. Sawfly larvae take up glucosinolates into their hemolymph from the gut where they are degraded to desulfoglucosinolates by glucosinolate sulfatase and subsequently sulfated at the glucose moiety by means of sulfotransferases (Opitz et al. 2011). Since excess glucosinolates are brought back into the gut and excreted via the frass, it is highly adaptive to earlier conversion within the hemolymph, since these modified glucosinolates in the gut can no longer be hydrolyzed by the remaining plant β-thioglucosidases (Müller 2009; Opitz et al. 2011).

4.4.2.5 Sequestration

Sequestration of plant chemical defenses is another method of insect adaptation to host plant chemistry (Opitz and Müller 2009). In many cases, it has been proven that insects exhibit better defense against their natural enemies after sequestration of defense compounds (Opitz and Müller 2009). For successful sequestration of glucosinolates, insect herbivores should not only possess some form of active uptake mechanism but also need to be capable to avoid glucosinolate hydrolysis. In theory, this could be accomplished by two principal ways: first, the compartmentalization of the glucosinolate-myrosinase system is not disturbed (as in the case of phloem-feeding aphids), and second, the uptake of intact glucosinolates is faster than their hydrolysis by myrosinases. This would require a relatively efficient transport mechanism and/or inhibition of myrosinase. In none of the herbivores that sequester glucosinolates has the precise mechanism of glucosinolate uptake from the gut lumen been reported; however, successful sequestration of intact glucosinolates has been established in both sucking and chewing herbivores. This suggests that both principle methods of retaining intact glucosinolates are operative in insects. To keep away from autotoxicity, a storage site is needed wherein breakdown of glucosinolates is averted till it is needed for defense (Müller 2009). Some insect species synthesize their own myrosinases, saved in a separate compartment away from the sequestered glucosinolates, which can be utilized for activating defense against predators (Beran et al. 2014; Francis et al. 2002).

B. brassicae (*Brassica* specialist) acquires glucosinolates in the hemolymph after phloem feeding with little or no cell disruption. The concentration of glucosinolates in hemolymph is higher in apterous individuals than alates which excrete large amounts of glucosinolates in the honeydew (Kazana et al. 2007). This insect is also known to synthesize its own myrosinase (distinct from plant myrosinase) which is stored in microbodies in flight (thorax) and head muscles (Beran et al. 2014). Endogenous myrosinase has also been reported in turnip aphid, *Lipaphis erysimi* (Bridges et al. 2002). The sequestered glucosinolates not only protect the aphids from attack of predators but also enhance growth and development of aphids. Generation time and fecundity of *Brevicoryne brassicae* correlate positively with concentration of total host glucosinolates (Chaplin et al. 2011; Kos et al. 2012), while composition of glucosinolates in aphid host plants and quantity sequestered can negatively affect the survival of both hoverflies and ladybugs (Francis et al. 2000, 2001; Kazana et al. 2007; Chaplin et al. 2011). Further, glucosinolate sequestration in *L. erysimi* releases isothiocyanates which synergize the action of aphid alarm pheromone *E-β-farnesene* required to initiate aphid dispersion after enemy attack (Dawson et al. 1987). Similar to aphids, flea beetle, *Phyllotreta striolata*, is also known to sequester plant glucosinolates and produce their own myrosinase (the so-called walking mustard oil bomb) (Kazana et al. 2007). The isothiocyanates produced after glucosinolate hydrolysis was found to have pheromone-like activities and set off aggregation behavior in adult beetles at high concentrations (Beran et al. 2011).

Sawfly larvae of the genus *Athalia* are also known to sequester glucosinolates from their host plants (Müller 2009; Opitz et al. 2010). Larval feeding is assumed to destroy the compartmentalization of the glucosinolate-myrosinase system, but larvae acquire intact glucosinolates in their hemolymph in concentrations higher than those in their food (Müller et al. 2001). When attacked by predators, these larvae release droplets of hemolymph (easy bleeding disruption of integument upon touch by predator), and the glucosinolates in the hemolymph probably act as deterrents to predators (Müller et al. 2002). However, so far it is unclear how the larvae manage to take up glucosinolates from their food without glucosinolate breakdown. When the larvae were transferred between plants with different glucosinolate profiles, glucosinolates from the new food were present in the hemolymph after 30 min, while the glucosinolates from the previous food plant were infrequently detectable after 24 h (Müller and Wittstock 2005). However, the feces contained trace quantities of intact glucosinolates arguing for metabolism of glucosinolates before excretion (Müller et al. 2001). Sawfly larvae are known to detoxify glucosinolates to desulfoglucosinolates before excretion (Sect. 4.4.2.4).

4.4.2.6 Symbionts

Symbiosis is extremely important for ecosystems' structure and function. Among the different types of symbioses (mutualistic, commensal, and antagonistic), mutualism is ubiquitous in all types of ecosystems and plays crucial roles in performance

of groups. Among the various symbiotic associations, the most cohesive form is endosymbiosis, wherein one partner is a symbiont (typically microorganisms, together with archaea, bacteria, and fungi) that lives inside the body of and intimately interacts with the other partner called host (normally animals and plants). Some microbes are dangerous or even lethal to the host and are referred to as parasites/pathogens, while others support the host species and appear to be mutualists because of their adaptive metabolic capabilities. Usually, mutualists are known as symbionts, as an antonym for parasites/pathogens (McFall-Ngai et al. 2013). Mutualistic associations with bacteria occur in animals, plants, fungi, or even protists, among which insects are wonderful for the prevalence and excessive variety of the associations they form with symbiotic microorganisms. Insects that feed completely on restricted diets, consisting of plant sap, vertebrate blood, and woody materials, typically own symbiotic microorganisms (mostly bacteria) of their frame, in which the symbionts play pivotal roles in host metabolism by presenting vital nutrients (e.g., critical amino acids and B vitamins) that lack in their ingredients and/or via digesting food substances (Baumann 2005; Brune 2014). Symbiotic bacteria commonly show strict host tissue tropism and are localized in symbiotic organs, even though the localization sample varies among insect species and stages from extracellular in the body cavity to intracellular in specialized cells referred to as mycetocytes/bacteriocytes (Moran and Telang 1998). Those symbionts are normally exceeded from the mother to offspring via sophisticated mechanisms for vertical transmission, which include transovarial transmission, egg-surface contamination, and coprophagy (Salem et al. 2015). For many insect groups, the symbiont phylogeny perfectly mirrors the host phylogeny, indicating that symbiotic associations have been maintained through strict vertical transmission for a protracted length of evolution, resulting in strong host-symbiont interdependency: host insects are afflicted by critical health defects without symbionts, while symbiotic microorganisms are generally unculturable (Kikuchi et al. 2011).

In addition to these dietary metabolic roles, current studies discovered more diverse functions of symbiotic bacteria in insects. Symbiotic bacteria are involved in tolerance to excessive temperature, parasitoid resistance, pathogenic virus protection, toxin synthesis, hardening of cuticle, integument coloration, and even sex determination (Su et al. 2013; Pietri and Liang 2018). In addition to toxin-degrading symbionts, these symbiotic bacteria, unlike the above mentioned nutrient individuals, are not necessary for the growth and reproduction of the host insects. However, such microbial partners play pivotal roles inside the evolution of insects by assisting in tolerance to variation in heterogeneous environments, underpinning the enormous variety of insects in the terrestrial environment. The presence of gut symbionts, *Serratia*, *Providencia*, *Pectobacterium*, and *Acinetobacter*, of the Gammaproteobacteria in cabbage root fly *Delia radicum* is known to degrade the toxic isothiocyanates. Symbionts encode SaxA gene which detoxifies the isothiocyanates by isothiocyanate hydrolase (Welte et al. 2016).

4.5 Conclusion

Glucosinolates have gained special significance in the study of insect-plant interactions as they are found in a wide variety of plants. Moreover, the presence of glucosinolates in *Arabidopsis*, a model organism in genetic studies, has played an important role in developing plant defense models for insect attacks, as well as for various pests. The primary role of myrosinase glucosinolate system in Brassicales is to ensure the protection against pests. Further research is needed to discover the range of stimulatory glucosinolates that possess exclusive side chains. Although a few tests have been carried out on some insect species with moderate range of glucosinolates, they were inadequate to derive generalized structure- activity relationship. In the past decade, research efforts have largely focused on the effect of glucosinolates on insect-plant interactions rather than with other bioactive compounds of plants. For example, glucosinolates are known to interact with plant waxes which in turn have effect on insect-plant interaction.

In addition, the specifier proteins are produced via plant redirecting the spontaneous approaches to biologically less active derivatives. Of what benefit is it to a plant to lower the discharge of poisonous plant secondary metabolites? Similarly, insect adaptation of toxic glucosinolate breakdown products has been reported. Comparative studies on the feature and dynamics of the recently determined enzymatic mechanisms and the variations in their distribution in phylogenetic family as well as far-off insect species can shed light on these fundamental questions. While sequestration has been confirmed for a small number of specialist insects, the dynamics and the mechanism (transporter enzymes) of the sequestration of various glucosinolates and their effects at higher trophic levels warrant further research efforts. At the end, even though glucosinolates are one of the most studied classes of plant compounds in insect-plant interactions, we are far from understanding their precise role in such interactions. Understanding such interactions is a major challenge that requires the use of molecular, biochemical, and ecological techniques.

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