Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits

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Plants respond to herbivore attack through a complex and variable system of defense, involving different physical barriers, toxic chemicals, and recruitment of natural enemies. To fully understand the relative role of each type of defense, their synergisms, redundancies, or antagonisms between traits, a variety of methods of enquiry, commonly used in plant physiology and ecology, have been employed. By overexpressing or silencing genes of interest, it is possible to understand the specific role of a particular defensive molecule or mode of action. We argue, however, that these types of experiments alone are not enough to holistically understand the physiological as well as ecological role of plant defenses. We thus advocate for the use of a combination of methods, including genetic modification, quantitative genetics, and phylogenetically controlled comparative studies.

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Introduction

Upon attack by herbivores, individual plants rely on a matrix-like variety of defense mechanisms, involving physical barriers, toxic or anti-nutritive secondary metabolites, and/or recruitment of predators and parasitoids [1,2••,3,4]. Moreover, a plant's defense arsenal is dependent on a variety of genetic, ontogenetic, and environmental factors, which together shape the multivariate defensive phenotype and outcome of the interaction. It thus seems pointless to consider different types of resistance traits as inherent, independent, constant, or singular in their mode of action [5]. For example, in a classic study, Berenbaum and Neal [6] showed that the biochemical toxicity of the furanocoumarin xanthotoxin, found in most Apiaceae, is radically enhanced by the presence of tiny

amounts of another compound (myristicin) in the plant. Duffey and Stout [5] showed that the variety of toxic compounds in tomato plants (alkaloids, phenolics, proteinase inhibitors, and oxidative enzymes), act in a matrical reaction, affecting the herbivore during ingestion, digestion, and metabolism in a way that would be presumably reduced, if each of the compounds were ingested separately. In *Nicotiana attenuata*, the presence of nicotine increases the negative impact of proteinase inhibitors on herbivores compared with plants with nicotine silenced [7••]. Finally, non-lethal resistance traits that reduce herbivore growth rates may act synergistically with the attraction of natural enemies of herbivores by increasing the window of opportunity for predation or parasitism [8,9].

Although few, if any, plant resistance traits may act independently, attempting to identify the contribution of traits is crucial for (1) understanding any redundancy, synergism, or antagonism between the multiple strategies employed against herbivores, (2) revealing the specificity of resistance traits against different herbivores, and (3) understanding the selective forces generating chemical and biological diversity. Here we assess different approaches, with particular focus on the current trend to genetically manipulate plants, to unravel the relative role of particular traits or classes of resistance. Each methodology (Table 1—Electronic appendix, Boxes 1–3) has advantages and disadvantages. We evaluate each in terms of its feasibility, ecological and evolutionary relevance, and applicability to different questions. Given that no one approach best captures the mechanistic basis of resistance and its likely role in ecology or evolution; we argue that a combination of two or more methods of investigation is needed to provide strong inference on the relative role of different resistance traits (Figure 1).

Implicating specific plant traits as agents of defense

Molecular genetic approaches employed to address questions about plant defense against herbivores include the use of transcriptome or microarray analysis, mutants, and genetic manipulation. Plants for which parts of the genome have been sequenced and annotated can be used to understand the role of chemical defense traits by conducting comparative transcriptome analyses. Currently, comparative transcriptome analyses of plant defenses are used to detect differences in global expression when

Figure 1
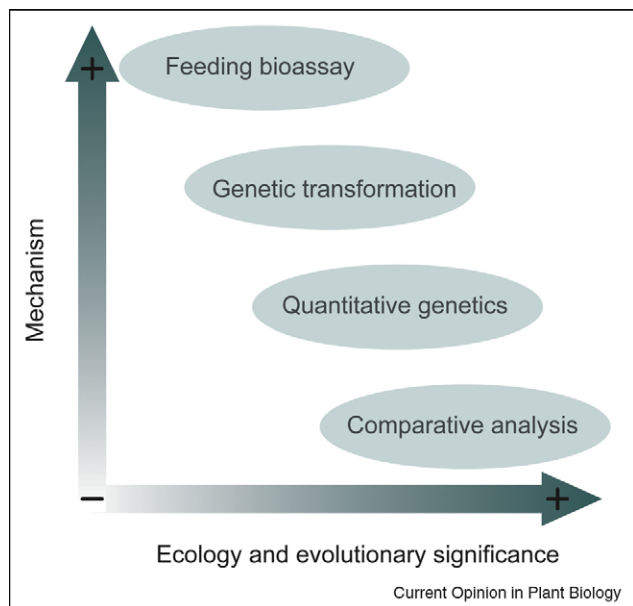
Schematic overview of the different approaches one can utilize to disentangle the relative role of plant defenses in light of mechanistic and evolutionary significance. We argue that each can be used in combination with the others to fully understand the physiological, as well as the ecological relevance of different plant defenses.

different herbivores and pathogens are attacking the plant (i.e. specificity of elicitation) [10,11], or to address questions about the responses of a particular gene of interest [12]. This approach is inherently correlative but has the advantage of taking a global perspective. Especially when comparing plants in different environments, this approach can be used to identify candidate genes for further, in-depth analysis or to suggest novel or previously unknown defense traits (e.g. [13,14]).

Concomitant with transcriptome analysis, the study of mutants via large screens, and subsequent comparison of phenotypes relative to a wild type, is currently used to detect important genes of interest. This was the case for COI1, first identified in *Arabidopsis* [15] and tomato [16]. Subsequently, homologs were sequenced and genetically silenced in *N. attenuata* [17] and rice [18]. After identification, the functional analysis of those genes further elucidates the role of defense traits. For example, by comparing wild types with jasmonic acid insensitive tomato mutants, Thaler *et al.* [19] demonstrated the concerted control of both direct and indirect defenses via the jasmonate pathway. Later, it was possible to demonstrate that in response to attack, jasmonoyl–isoleucine stabilizes the interaction between COI1 and JAZ proteins. The JAZ proteins are then destroyed, liberating the transcription factors of genes that produce proteins involved in defense and development, as well as of *JAZ* genes to restrain the jasmonate response [20[••],21[••]].

In *Arabidopsis*, to explore the role of two different myrosinases, enzymes responsible of the breakdown of glucosinolates and producing toxic, anti-herbivore compounds, Barth and Jander [22], identified double mutants, and showed redundancy in the activity of the two enzymes against specialist and generalist chewing herbivores. Another example of how *Arabidopsis* mutants have been used to study different defense traits comes from a study by Chehab *et al.* [23]. By generating an ensemble of plant genotypes lacking either jasmonates, C6 aldehydes, or both, it was shown that jasmonates were more strongly associated with direct defenses, whereas C6 aldehyes and hexenyl acetate were predominant signals involved in indirect defenses.

The benefits of the mutant approach are that it can be applied to any plant system and can identify the specific role of particular genes or pathways. The downside of this approach, however, is that it is (1) remarkably labor intensive to conduct mutant screens and (2) may be difficult to pinpoint the specific mutation.

Plant genetic transformation generally utilizes two methodologies: (1) insertion of foreign genes into a plant genome or (2) overexpression or silencing of endogenous genes. Currently, the genetic transformation of plants aimed to disentangle the relative roles of defenses in plant–insect or plant–pathogen interactions involves species in four families (Solanaceae, Brassicaceae, Poaceae, and Salicaceae), including the tobaccos *Nicotiana* spp., tomato *Solanum lycopersicum*, potato *S. nigrum*, pepper *Capsicum* spp., *Arabidopsis thaliana*, corn *Zea mays*, rice *Oryza* spp., and poplar *Populus* spp.

Some of the best examples of using genetic transformation to study defense come from a study of *N. attenuata*, where both trypsin proteinase inhibitors (TPI) and nicotine expression, two major types of direct defenses in those plants, were independently and simultaneously silenced [7]. Results showed that TPI alone conferred no resistance to the herbivore *Spodoptera exigua*, but in combination with nicotine, TPI contributed synergistically to the defensive response [7]. In addition, Mitra *et al.* [14] elegantly showed defensive functions of induced proteins with previously unknown functions in *N. attenuata*. Silencing genes coding for proteins of primary metabolism, generally upregulated by herbivory, tended to benefit larval performance. However, silencing genes of typically downregulated proteins tended to decrease insect performance, whereas neither proteinase inhibitors nor nicotine was associated with reduced larval performance. This lack of consistency between experiments underscores the matrix-like nature of plant defense: even when experiments are conducted with the same lineages of plants under highly controlled conditions, some of our best examples of 'defense' traits fail to mediate resistance under some conditions. Redundancy, synergy, and

antagonism are the rule, and even micro-environmental contingency should be expected.

Transformation of plants with foreign genes responsible for the production of secondary metabolites is a promising technique to understand the role of plant volatiles as attractants of natural enemies of herbivores. Under laboratory conditions, Schnee *et al*. [24] showed that the overexpression of corn (*Zea mays*) terpene synthases in *Arabidopsis* rendered the plant attractive to wasps that were trained to respond to terpenes released by corn plants. Furthermore, Kollner *et al*. [25] identified genes coding for the production of (*E*)-β-caryophyllene in the roots of corn. This compound is involved in the attraction of predatory nematodes to corn roots damaged by rootworms *Diabrotica v. virgifera* [26]. Given that the expression of this gene after herbivore attack was lost during the breeding process in most of the North-American varieties of corn, genetic restoration of these products could result in increased biological control of corn rootworms.

Although at first blush, planting genetically modified plants in the field to test ecologically relevant questions may sound like an oxymoron, we believe that such research has led to increased and important knowledge of the mechanisms of specific plant–herbivore interactions. Nonetheless, it is the evolutionary relevance of such studies that remains unclear. Limitations include

(1) The expression of a particular gene depends on genetic background (i.e. epistasis) [27]. (2) The function of a particular gene may be dependent on other traits (as demonstrated in the excellent recent studies that manipulate two factors, discussed above). Here, however, the question remains: how many traits or genes are necessary to factorially manipulate in order to fully understand redundancy or synergism between traits? (3) Evolutionary arguments are often made about the fitness value of particular genes, yet most evolution may occur gradually on quantitative (i.e. continuous) traits, and (4) Ecological implications of major genetic transformations may be similarly overstated. Although the effect of altering a gene/pathway may indeed have an effect, whether that gene functions in this way in nature is usually unknown. Variation in the natural populations usually will not contain such dramatically altered plants (and thus the behavior of interacting organisms will be relatively extreme, and without the context, time of acclimation, or adaptation to the new phenotype).

One more classical genetic approach to address some of the ecological and evolutionary deficiencies of molecular approaches is quantitative genetics. This approach requires the knowledge of sib relationships between individuals of the same species or populations. Besides being a tool to understand the heritable genetic variation, covariation be-tween traits, and micro-evolutionary processes, is it a relative simple methodology to work with in the laboratory and field. Moreover, it is possible to test for natural selection by comparing population differentiation for neutral molecular loci estimated by F_{ST} and for the additive genetic component of quantitative traits estimated by Q_{ST} [31]. The disadvantages, however, are that it is almost impossible to nail specific mechanisms behind the observed pattern. Targets of natural selection can be identified [32,33], costs and benefits of traits can be estimated in a real-time evolutionary framework, but correlated traits may always be responsible for some of the effect.

Synthesis

It has been estimated that the diversity of secondary metabolites in the ~350 000 extant flowering plant species probably exceeds 200 000 distinct compounds [2[••]]. Despite the fact that it is very unlikely that most of these secondary compounds are a mere result of primary metabolism and are waste products, and that it is of common agreement that they evolved as defenses against herbivores or pathogens, the question of their specific functions remains largely unknown. To summar-

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Box 1 Quantitative genetics.

By studying within and between population variation of plants in one or more defense-related traits, it is possible to infer the heritability of traits, covariation of a trait with others, and identify natural selection on the trait [32]. Of course, what is observed is the combined effect of all genes involved in generating the phenotype. Nonetheless, we argue here that the use of quantitative genetic experiments is a valuable tool to disentangle the roles of various traits in resistance, especially in the context of the real micro-evolutionary processes that shape defensive traits against herbivores. Quantitative genetic experiments with wild populations of *A. lyrata* and analyses of phenotypic and genetic correlations between candidate defense traits and insect resistance suggested that *A. lyrata* resistance was conferred by a combination of indole glucosinolates and trichome density and, to a lesser extent, aliphatic glucosinolate ratios and leaf thickness [39[•]]. The lack of a genetic correlation between the two traits allowed for the independent assessment of their impact.

Quantitative genetic experiments can be useful to address questions of specificity of interactions. For example, *Asclepias syriaca* plants were used to test the relationship between a series of resistance traits and selection by five specialist herbivores. Results highlighted directional selection favoring resistance to herbivory, latex production, and nitrogen content of the leaves. Moreover, trichomes and latex were each negatively genetically correlated with abundances of herbivores, but not with herbivore damage (or each other); cardenolides and induced plant resistance were negatively genetically correlated with growth of monarch caterpillars *Danaus plexippus* [32].

A more recent addition to the study of quantitative genetics, quantitative trait loci studies (QTLs, which identify stretches of DNA that are closely linked to the genes that underlie the trait in question), have mapped genetic differences in expression of defense compounds [40] or between different population of plants that have taken different evolutionary trajectories [41]. In particular, population variation can be exploited to study natural selection on traits by examining traits and their interactions [42] or by examining the ratio of divergence between populations at neutral loci compared to phenotypic divergence in putatively adaptive traits [31].

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ize the debate around the evolutionary and ecological significance of defense compounds, two antagonistic views prevail: (1) Most secondary chemicals serve no role other than to generally contribute to the non-adaptive (but necessary) variability to increase the probability of producing a few biologically active compounds to use when ecological circumstances requiring defense arise or (2) phytochemical diversity within species is itself adaptive [28]? We have here summarized different approaches taken to implicate the function of particular compounds as defense against herbivores (Figure 1). By genetically manipulating plants through the silencing, overexpressing, or introducing foreign genes into plants, it is possible to address specific questions at the gene or compound level. The still very young area of genetic manipulation to address mechanisms of plant defenses has great advantages in resolving specific mechanisms [7,22,29,30].

Nonetheless, it is almost impossible to address significant questions on the evolution or adaptation of defense-related traits with the genetic manipulation method alone. For such analyses, quantitative genetic experiments (Box 1), diet experiments (Box 2), and phylogenetic controlled comparative analysis (Box 3) are well suited. A quantitative genetics study could be coupled with genetic manipulation or pharmacological study to complete the circle of evolutionary ecology to mechanism. For example, The ESP locus in Arabidopsis was discovered as natural genetic variation in glucosinolate breakdown to nitriles or isothiocyanates (QTL mapping) [34]. Effects of these have been studied in diet experiments [35] and with mutants [36]. Similarly, Steppuhn *et al.* [37] found a rare mutant in a natural population of *N. attenuata* lacking TPI

Box 2 Diet studies.

Creating artificial blends of toxic compounds is useful to study the relative role of chemical diversity (Figure 2). For example, the toxicity of a group of pyrrolizidine alkaloids (PAs) commonly found in *Senecio* spp. was tested on five generalist herbivores; the compounds were first isolated and then were added alone or in a mixture to an artificial diet. The relative effects of PAs differed for the insect species, and it was suggested that such specificity could contribute to the evolution of diversity of compounds [43]. Moreover it was shown that the same compound, the alkaloid retrorsine, was more active in the free-base PA form over the oxide form.

Diet studies are also a powerful tool to investigate synergism and redundancy. For example, mixtures of three *Piper* amides had the most dramatic deterrent effect on generalist and specialist herbivores [44]. Diet studies have also been focusing on the interaction between different classes of compounds. The presence of tannic acid in diet strongly affected grasshopper dietary regulation on different C:N ratio diets [45].

Subtraction of defensive compounds also holds promise as a tool to manipulate natural blends. For example, D'Alessandro and Turlings [46] argued that chemically filtering (and subsequently restoring) the original blend of volatile organic compounds released by a damaged plant could be used to determine the compounds that are perceived and learned during parasitoid foraging.

Figure 2

Current Opinion in Plant Biology

Artificial diets can be used to understand synergism, additivity, or antagonism between nutritive or toxic plant compounds. Shown here is a monarch *Danaus plexippus* caterpillar feeding on an agar-based diet complemented with ground milkweed leaves.

defenses; the genetic basis and impacts of this mutant were comparable to transformed plants. Studies of natural selection on that mutant could complement the knowledge gathered from more controlled studies.

Genetic and diet studies have similarly lead to complementary beneficial results. For example, the use of

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Box 3 Comparative approaches to studying plant resistance.

Historically, species were compared as independent units to correlate traits with herbivory or performance of herbivores. For example, Coley [47], compared damage imposed by herbivores on a large set of coexisting tropical tree species and proposed hypotheses on how selection may have favored different defense investments in species with different life-histories and habitat affinities. Especially in large-scale studies like this one, it is unlikely that correlated traits were causing the effects observed because such a large diversity of taxa with different evolutionary histories was studied. With the advent of phylogenetic information, such correlations can be made even stronger by factoring out any impact of shared evolutionary ancestry in trait similarity (this is the hallmark of the phylogenetic independent contrast) [1]. For example, in the milkweed family, the repeated evolution of trichomes (either increases or decreases) is associated with evolution of latex exudation and associated changes in monarch caterpillar growth [48••].

Additionally, the study of phylogenies and defense can help make inferences on macro-evolutionary processes that have shaped defensive strategies within clades or communities. For example, clade-level predictions of 'escalation' were predicted by classic plant defense theory [1]. Although a phyletic decline in key resistance traits (cardenolides, latex and trichomes) was observed, escalation in tolerance (the ability to regrow) and phenolics was also seen in 34 milkweeds [49] (Figure 3). At the community level, Becerra [50••] has recently shown that coexisting *Bursera* spp. tend to be more chemically dissimilar than is expected by chance. The dominant herbivores, *Blepharida* spp. beetles tend to prefer chemically similar plants, suggesting that herbivores are strong selective forces on plant secondary chemistry at the community level [50••].

Figure 3
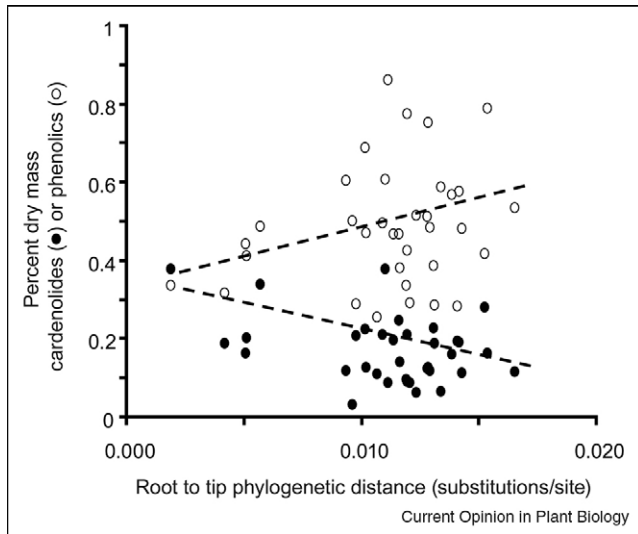
Consistent with the defense escalation hypothesis, the diversification of the milkweeds was associated with a trend for increasing phenolic production; this pattern was reversed for cardenolides, toxins sequestered by specialist herbivores. Each dot represents an *Asclepias* species that was grown from seed in a common environment ($n = 5$ replicates per species). The *X*-axis indicates the level of molecular divergence (at neutral loci) since the hypothesized ancestor. Dashed lines indicated phylogenetically corrected significant slopes. Modified from Agrawal *et al.* [49].

two *Arabidopsis* mutants, differing in the production of myrosinase activities, combined with feeding bioassays, showed that the post-ingestive breakdown of indole glucosinolates provides a defense against aphids that can avoid glucosinolates activation by plant myrosinases [38]. The consistent use of two or more lines of evidence (Figure 1) will undoubtedly result in a clearer picture of defense.

A plant's response to herbivore attack is generally so complex that establishing the relevance of a particular trait for the interaction is often difficult. The studies reviewed above employ different techniques to address a similar question: how can we disentangle the relative roles of different plant defenses? By a combination of two or more of such methodologies, we can obtain simultaneous information on the mechanisms and the ecological or evolutionary significance of the defensive trait.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbi.2009.05.005.

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Table 1

References	Model plant	Methodology	Defense type	Tools required	Eco	Evo
Genetic manipulation						
[1]	*A. thaliana*	mutant screening	glucosinolates-myrosinase complex	recombinant lines	No	No
[2]	*A. thaliana*	genetic manipulation	direct and inidirect defenses	mutants	No	No
[3]	*Lycopersicum*spp.	genetic transforamtion	Proteinases	genetic manipulation	No	No
[4]	*A. thaliana*	genetic transforamtion	JA system	genetic manipulation	No	No
[5]	*A. thaliana*	trascriptome analysis	all possible	genome sequences	No	No
[6]	*Nicotiana attenuata*	genetic transformation	direct and inidirect defenses	modified plants	Yes	No
[7]	*A. thaliana*	recombination	glucosinolates-myrosinase complex	mutants	No	No
[8]	*Z.mays*	gene expression	indirect defense	gene sequences	No	Yes
[9]	*A. thaliana*	microarrays RT-PCR	glucosinolates-myrosinase complex, phyotohormones	RI and microarrays	No	No
[10]	*N. attenuata*	genetic tranformation	proteine involved in primary metabolism	genetic manipulation	No	No
[11]	*N. attenuata*	mutant comparison	COI1	mutants	Yes	No
[12]	*Z. mays* and A. *thaliana*	genetic transforamtion	terpenes	modified plants	No	No
[13]	*N. attenuata*	genetci transformation	direct and inidirect defenses	mutants	No	No
[14]	*N. attenuata*	transformation	Trypsin proteinase inhibitor and nicotine	modified plants	Yes	No
[15]	*L. esculentum*	mutant comparison	JA system	mutants	Yes	No
[16]	*A. thaliana*	genetic transforamtion	JA system	genetic manipulation	No	No
Quantitative genetics						
[17]	*Lupinus* spp.	Quantitative genetic analysis	alkaloids reistance	siblings	Yes	Yes
[18]	*Asclepias syriaca*	Quantitative genetic analysis	cardenolides, latex, trichomes, leaf toughness and N	families of plants	Yes	Yes
[19]	*A. thaliana*	genetic families	glucosinolates diversity	gentoypes	Yes	Yes
[20]	*Brassica rapa*	Quantitative genetic analysis	glucosinolates trypsin inhibitors resistance	sibs	Yes	Yes
[21]	*A. lyrata*	Quantitative genetic analysis	glucosinolates trichomes resistance	families, sibs	Yes	Yes
[22]	*Eucalyptus* spp.	QTL analysis	Formylated phloroglucinols	clones	Yes	No
[23]	*B. oleracea*	different populations	different glucosinolates, resistance	populations genotypes	Yes	Yes
[24]	*A. thaliana*	Quantitative	trichomes and	genotypes	Yes	Yes

		genetic analysis	glucosinolates			
Diet						
[25]	artificial diet	feeding bioassays	tannic acid, protein:carbon ratio	chemical extratracts, diet	Yes	No
[26]	*Conium maculatum*	feeding bioassays	PAs	chemical extracts	Yes	Yes
[27]	*Roldana barba-johannis*	feeding bioassays	sargachromenol, sargahydroquinoic acid, and sargaquinoic acid	chemical extracts	No	No
[28]	*Z. mays*	foraging bioassays	VOCs indirect defenses	chemical and physical filtering	No	No
[29]	PAs bearing plants	feeding bioassays	PAs	chemical extracts	Yes	No
[30]	*Piper spp*	feeding bioassays	piper amides	chemical extracts	Yes	No
[31]	Piper spp	comparative analysis	chemical, physical and indirect defenses	coexisting plants of same genus	Yes	No
[32]	artificial diet	feeding bioassays	phytic acid and xanthotoxin	chemical extracts and diet	No	No
[33]	*Asteraceae sp*	feeding bioassays	Sesquiterpene Lactones and phototoxin	chemical extracts	Yes	No
[34]	*Adenostyles* spp.	feeding bioassays	Pas and sesquiterpenoids	chemical extracts	Yes	Yes
[35]	different algal species	feeding bioassays	secondary chemistry and physical defense	chemical extracts	Yes	No
[36]	lactiferous plants	feeding bioassays	latex, cysteine proteases	chemical (proteases, prot. Inhibit)	No	No
[37]	Inga spp	chemical comparison	phenolics	two species	Yes	No
[38]	*Neomeris annulata*	feeding bioassays	secondary chemistry (terpenoids)	chemical extracts	No	No
[39]	PAs bearing plants	feeding bioassays	PAs	chemical extracts	Yes	No
[40]	*Salix spp*	feeding bioassays	secondary chemistry and salicilates	chemical extracts	Yes	No
[41]	*Lactuca sativa*	feeding bioassays	latex and chemical extracts of latex	susceptible and resistant varieties	No	No
[42]	*Gossypium hirsutum*	feeding bioassays	(+/-) gossypol	chemical extracts	No	No
Comparative						
[43]	*Asclepias* spp	comparative analysis	resistance and tollerance related traits	phylogeny, common garden experiment	No	Yes
[44]	*Bursera* spp	comparative analysis	co-evolution	phylogeny	Yes	Yes
[45]	*Bursera* spp	comparative analysis	chemical and physical defenses	phylogeny	Yes	Yes
[46]	flowering plants	phylogenetic analysis	diversity	phylogeny	No	Yes
[47]	*Acacia* spp.	comparative analysis	constitutive *vs.* induced defenses	phylogeny	Yes	Yes
[48]	different plants	comparative	leaf toughness and	feeding assays	Yes	No

		analysis	trichomes			
[49]	*Hakea* spp.	comparative analysis	chemical (phenolics) and physical defenses	coexinsting species of same genera	Yes	No
[50]	*Gossypium* spp.	comparative analysis	constitutive, induced defenses	phylogeny	No	Yes
[51]	Brassicaceae	phylogenetic analysis	co-evolution	phylogeny	No	Yes

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