Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy[1][OPEN]

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The plant kingdom produces hundreds of thousands of low molecular weight organic compounds. Based on the assumed functions of these compounds, the research community has classified them into three overarching groups: primary metabolites, which are directly required for plant growth; secondary (or specialized) metabolites, which mediate plant–environment interactions; and hormones, which regulate organismal processes and metabolism. For decades, this functional trichotomy of plant metabolism has shaped theory and experimentation in plant biology. However, exact biochemical boundaries between these different metabolite classes were never fully established. A new wave of genetic and chemical studies now further blurs these boundaries by demonstrating that secondary metabolites are multifunctional; they can function as potent regulators of plant growth and defense as well as primary metabolites sensu lato. Several adaptive scenarios may have favored this functional diversity for secondary metabolites, including signaling robustness and cost-effective storage and recycling. Secondary metabolite multifunctionality can provide new explanations for ontogenetic patterns of defense production and can refine our understanding of plant–herbivore interactions, in particular by accounting for the discovery that adapted herbivores misuse plant secondary metabolites for multiple purposes, some of which mirror their functions in plants. In conclusion, recent work unveils the limits of our current functional classification system for plant metabolites. Viewing secondary metabolites as integrated components of metabolic networks that are dynamically shaped by environmental selection pressures and transcend multiple trophic levels can improve our understanding of plant metabolism and plant–environment interactions.

Plants can use simple, inorganic precursors to synthesize a large diversity of low M_r organic compounds. This synthetic capacity helps plants to colonize diverse and challenging environments. Low M_r organic compounds are commonly separated by perspective function into primary metabolites, secondary metabolites (also called specialized metabolites or natural products), and plant hormones (Fig. 1; Taiz et al., 2015). Primary metabolites are highly conserved and directly required for the growth and development of plants (Fernie and Pichersky, 2015). Secondary metabolites, including major groups such as phenolics, terpenes,

ADVANCES

- Genetic and chemical experiments demonstrate that plant secondary metabolites can act as regulators of development, growth, and defense.
- Secondary metabolites can be re-integrated into plant primary metabolism, thus acting as primary metabolites *sensu lato*.
- Many secondary metabolites have multiple functions, and these functions are reflected in corresponding spatiotemporal and stress-dependent accumulation and structural variation patterns.
- The multifunctionality and metabolic integration of secondary metabolites is mirrored in the adaptations of specialized herbivores.

and nitrogen-containing compounds, are often lineage specific and aid plants to interact with the biotic and abiotic environment (Hartmann, 2007). Finally, plant hormones are defined as small compounds that regulate organismal processes, including the production of the other metabolites, by interacting with receptor proteins (Davies, 2004).

Despite the fact that definitions of secondary metabolites are inherently diffuse (Hartmann, 2007; Pichersky and Lewinsohn, 2011; Davies, 2013), the distinction between primary metabolites, secondary metabolites, and plant hormones has found its way into textbooks and shapes our thinking in plant biology to this day. An illustrative example is the field of plant–herbivore interactions, where major efforts have gone into disentangling how plants protect their primary metabolites (serving as nutrients for herbivores) using secondary metabolites (serving as defenses for plants), and how adapted herbivores manage to extract primary metabolites while avoiding the negative effects of secondary metabolites (Awmack and Leather, 2002; Howe and Jander, 2008; Zhou et al., 2015; Erb and Reymond, 2019). In this context, plant hormones are investigated as regulators of primary and secondary metabolism, defense, and resistance that may be manipulated by adapted herbivores (Howe and Jander, 2008; Schuman and Baldwin, 2016; Stahl et al., 2018), similar to pathogens (Kazan and Lyons, 2014). The biochemical coevolutionary arms–race theory (Ehrlich and Raven, 1964), a key concept in plant–herbivore interactions (Berenbaum and Zangerl, 2008; Jander, 2018), postulates

that plant secondary metabolites evolve in response to herbivore pressure, resulting in the evolution of resistance mechanisms in herbivores. The resulting arms race is thought to drive the diversity of plant secondary metabolites and insect herbivores (Futuyma and Agrawal, 2009).

Over the last decades, the distinction between primary metabolites, secondary metabolites, and plant hormones has proven a useful approximation. However, the emergence of a more detailed understanding of plant metabolism may require us to revisit this functional partitioning (Neilson et al., 2013; Maag et al., 2015; Kliebenstein, 2018; Pichersky and Raguso, 2018; Zhou et al., 2018). In particular, an increasing number of genetic and functional studies on plant secondary metabolites are blurring the functional trichotomy by showing that plant secondary metabolites can have regulatory functions and serve as precursors for primary metabolites. In this review, we discuss this evidence, mostly focusing on examples that rely on the use of natural knockout variants, mutants, and transgenic plants altered in their capacity to produce certain secondary metabolites in combination with chemical complementation assays to demonstrate activity of the metabolites. We illustrate that for an increasing number of plant secondary metabolites, a strict functional separation from regulators and primary metabolites may not do them justice and possibly hinders our progress in understanding their roles for plant survival in hostile environments.

Figure 1. Low molecular weight compounds in plants are functionally classified as primary metabolites, secondary metabolites, or hormones. Present work on plant secondary metabolites demonstrates that many of them also have regulatory roles, and some are demonstrated precursors of primary metabolites. Note that primary metabolites and hormones also show functional overlap with the other metabolite classes (not discussed here). These findings blur the functional trichotomy of plant metabolism and call for a reassessment of ecological and evolutionary frameworks that are based on this model.

INTEGRATION OF PLANT SECONDARY METABOLITES INTO REGULATION AND METABOLISM

Early Evidence for Metabolic Integration of Secondary Metabolites

In 1977, David Rhoades studied the properties of creosotebush (*Larrea* spp.) leaf resin. He found that the resin, which contained high levels of phenylpropanoid derivatives (lignans), absorbed ultraviolet radiation, reduced evaporative water loss across cellulose membranes, and had the capacity to form complexes with proteins, thus possibly reducing the digestibility of plant materials for herbivores (Rhoades, 1977, p. 281). Rhoades (1977) thus postulated that "…any chemical system possessed by a plant must necessarily be integrated into the total metabolic scheme and multiple functions are to be expected." In other words, Rhoades (1977) proposed that secondary metabolites are not end points, but integrated components of plant metabolism, and may, by consequence, take on any number of functions, similar to other plant metabolites. Indeed, evidence was emerging at that time that secondary metabolites may regulate growth and defense, as exogenously applied flavonoids could modulate polar auxin transport and catabolism (Stenlid, 1963; Stenlid, 1976), glucosinolate breakdown products could replace
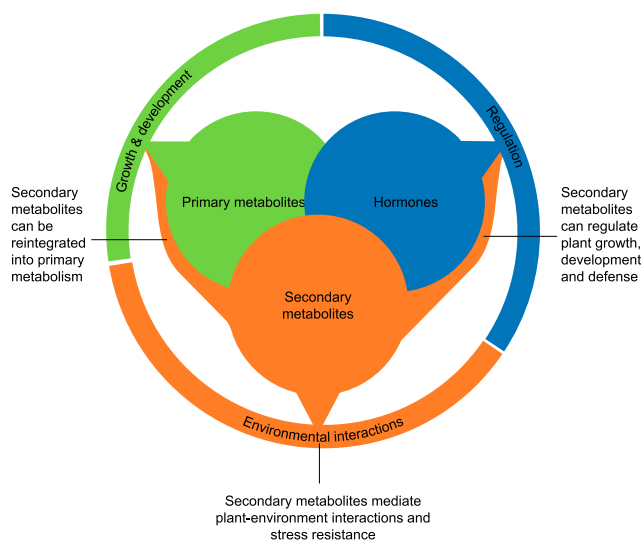
auxins in inducing hypocotyl bending (Hasegawa et al., 1986), and induced volatiles promoted resistance and defense regulation in neighboring trees (Baldwin and Schultz, 1983; Rhoades, 1983).

Secondary Metabolites as Regulators of Plant Defense

Following early preliminary evidence of secondary metabolites regulating defenses, genetic evidence followed in 2009, when it was reported that Arabidopsis (*Arabidopsis thaliana*) mutants defective in indole glucosinolate biosynthesis no longer mount a callose defense response following Flg22 treatment. Callose formation is rescued by adding 4-methoxy-indol-3-ylmethylglucosinolate (Clay et al., 2009). The myrosinase *PEN2* is required for this phenomenon, implicating glucosinolate breakdown in callose regulation (Clay et al., 2009). Shortly thereafter, it was discovered that indole-derived benzoxazinoid secondary metabolites have a comparable callose regulatory function in cereals. Benzoxazinoid-deficient *bx1* maize (*Zea mays*) mutants are defective in aphid- and chitosan-induced callose deposition, and callose induction is rescued by the addition of DIMBOA or DIMBOA-Glc (Ahmad et al., 2011; Meihls et al., 2013). In both cases, the capacity to regulate callose is structurally specific and depends on the modification of the indole-derived ring. In Arabidopsis, indol-3-ylmethylglucosinolate, which lacks a methylated hydroxy-group on the aromatic ring, is inactive, whereas the methylated form is active (Clay et al., 2009). In maize, DIMBOA-Glc, which lacks a methylated hydroxy-group at the nitrogen, is active, whereas the methylated form (HDMBOA-Glc) is inactive (Li et al., 2018a). Whereas the callose response to benzoxazinoids is conserved between wheat (*Triticum aestivum*) and maize, they do not elicit callose in Arabidopsis, and intact glucosinolates do not elicit callose in maize (Li et al., 2018a). These studies show that callose regulation by secondary metabolites is highly specific, tightly controlled, and likely evolved repeatedly. The mechanism underlying secondary metabolite–induced callose formation awaits to be elucidated. Glucosinolates and benzoxazinoids may, for instance, promote callose production by regulating hormonal pathways (Burow et al., 2015; Katz et al., 2015), through transcriptional regulation (Kim et al., 2015), or by directly initiating callose formation posttranslationally.

Interestingly, glucosinolates and benzoxazinoids also seem to regulate the accumulation of other secondary metabolites (Hemm et al., 2003; Kim et al., 2015; Li et al., 2018a). In Arabidopsis, mutants that are defective in the atypical myrosinase PEN2 release lower amounts of Trp-derived metabolites such as camalexin upon flg22 treatment (Frerigmann et al., 2016) and infection by *Pseudomonas syringae* (Stahl et al., 2016). Furthermore, mutants defective in the CYP83B1 enzyme required for indole glucosinolate production also show lower accumulation of the phenylpropanoid

sinapoylmalate (Kim et al., 2015). The phenylpropanoid phenotype is rescued in mutants that no longer produce the substrate of CYP83B1, indole-3-acetaldoxime (Kim et al., 2015), suggesting that it may be the aldoxime overaccumulation rather than the lack of downstream glucosinolates that suppresses sinapoylmalate. Suppressor screens showed that the phenylpropanoid phenotype is also absent in plants that have mutated *MEDa/b* genes, which encode key components of a large multisubunit transcriptional complex that regulates phenylpropanoid biosynthetic genes (Kim et al., 2015; Dolan et al., 2017). A recent study demonstrates that a group of *Kelch Domain F-Box* (*KFB*) genes that are involved in PAL inactivation (Zhang et al., 2013) are upregulated in indole glucosinolate mutants in a MED5-dependent manner, whereas PAL-activity is suppressed (Kim et al., 2020). PAL-activity and sinapoylmalate accumulation are (partially) rescued in glucosinolate-deficient KBF mutants (Kim et al., 2020). The model emerging from these studies is that aldoximes, which accumulate in CYP83B1 mutants, increase KFB-mediated PAL degradation through MED5 transcriptional regulation as well as other, yet unknown, mechanisms (Kim et al., 2015; Kim et al., 2020). As aldoximes are produced by many different species, this form of defense regulation may also occur beyond glucosinolate-producing plants (Kim et al., 2020). Interestingly, wheat lines overexpressing a maize benzoxazinoid *O*-methyl transferase and thus accumulate more HDMBOA-Glc and less DIMBOA-Glc also show higher levels of the phenylpropanoid ferulic acid, despite unaltered pool sizes of amino acid precursors (Li et al., 2018a), suggesting that phenolic compounds may also be regulated by other secondary metabolite pathways.

Apart from glucosinolates and benzoxazinoids, volatile secondary metabolites such as terpenoids, green-leaf volatiles, and aromatic compounds can also regulate plant defenses (Baldwin et al., 2006; Godard et al., 2008; Erb, 2018; Bouwmeester et al., 2019). Many of these volatiles are released upon herbivore- or pathogen attack and are capable of directly inducing or priming hormonal defense signaling pathways and resistance. In maize, for instance, mutants that are defective in their capacity to produce volatile indole are unable to prime their systemic tissues to rapidly release terpenes upon herbivore attack (Erb et al., 2015). Adding indole to the headspace of maize plants restores this priming phenotype (Erb et al., 2015). Rice (*Oryza sativa*) plants also respond to indole through priming of early defense signaling elements such as the map kinase OsMPK3 (Ye et al., 2019). Transgenic plants that are deficient in *OsMPK3* expression are no longer responsive to indole, suggesting that indole acts via the priming of early defense signaling (Ye et al., 2019). In Arabidopsis, *geranylgeranyl reductase1* mutants are defective in systemic acquired resistance against *P. syringae* (Riedlmeier et al., 2017). Adding the pathogen-induced volatiles α- and β-pinene to the headspace of the mutant restores resistance, with the response depending on intact salicylic acid signaling and the *AZELAIC ACID*

INDUCED (*AZI1*) gene (Riedlmeier et al., 2017). The precise role of other volatile secondary metabolites that can regulate defenses at physiological concentrations, including homoterpenes (Arimura et al., 2000) and green-leaf volatiles (Ameye et al., 2018), has not yet been explored using genetic approaches, but their activity has been demonstrated clearly through chemical complementation (Arimura et al., 2000; Engelberth et al., 2004; Frost et al., 2008; Meents et al., 2019). Further support for the potential regulatory role of defense volatiles comes from *LOX2*-silenced *Nicotiana attenuata* plants, which are deficient in the production of herbivory-induced, green-leaf volatiles. In contrast with the other systems where volatiles induce defense, the *LOX2* mutation leads to stronger expression of defense-related genes in neighbors than wild-type plants, suggesting that volatiles can also suppress defenses (Paschold et al., 2006).

In summary, at least five classes of secondary metabolites (glucosinolates, benzoxazinoids, terpenes, aromatics, and green-leaf volatiles) are now confirmed to act as potential regulators of in planta defense. It is exciting to speculate that there are many other secondary metabolites that play similar regulatory roles. An important gap of knowledge is the mechanism by which secondary metabolites regulate defenses. As many of the secondary metabolites are chemically reactive (Farmer and Davoine, 2007; Hadacek et al., 2010), it is possible that they act indirectly by depleting detoxification enzymes, thus triggering the accumulation of known signaling molecules such as reactive oxygen species (ROS; Khokon et al., 2011). However, as discussed below, secondary metabolites may also have hormone-like properties by binding to specific receptor proteins (Katz et al., 2015). More work on the targets of secondary metabolites in planta is clearly warranted and would help to clarify the ecological and evolutionary context of their capacity to regulate defenses.

Secondary Metabolites as Regulators of Growth and Development

Plants regulate their growth dynamically and often reduce their investment into growth and development upon herbivore- or pathogen attack. This reduction in growth is thought to be largely due to the reconfiguration of a plant's signaling network rather than a lack of resources (Kliebenstein, 2016; Machado et al., 2017; Guo et al., 2018). Strikingly, plant secondary metabolites and their breakdown products are being (re)-discovered as plant growth modulators, thus adding another layer of regulation to growth-defense patterns. Again, glucosinolates provide a mechanistic example of how secondary metabolites can modulate growth. When applied to the roots of Arabidopsis and many other plant species, the aliphatic 3-hydroxypropylglucosinolate inhibits root meristematic growth at physiological concentrations via an intact Target of Rapamycin pathway (Malinovsky et al., 2017). The exact molecular interaction partner of 3-hydroxypropylglucosinolate remains

unknown. Studies on the indole glucosinolate breakdown product indole-3-carbinol have identified an unexpected target protein. Indole-3-carbinol accumulates upon wounding in Arabidopsis and rapidly reduces root growth upon exogenous application. In vitro, indole-3-carbinol interferes with the interaction between auxin and its receptor TIR1 by binding at an allosteric site (Katz et al., 2015). As the indolic glucosinolate catabolite likely binds directly to TIR1 (Katz et al., 2015), one may argue that TIR1 acts as an indolic glucosinolate receptor that mediates the regulation of growth by a plant secondary metabolite. Another link to auxin signaling was found with a structurally unrelated aliphatic glucosinolate. This was found by the initial observation that the auxin-sensitive repressors IAA5, IAA6, and IAA19 strongly regulate 4-methylsulfinylbutyl glucosinolate (4-MSOB) levels in dehydrated Arabidopsis plants (Salehin et al., 2019). *Iaa5,6,19 mutants* fail to close their stomata upon drought stress, a phenotype that can be reverted by adding 4-MSOB (Salehin et al., 2019). Together with the finding that glucosinolate biosynthesis and activation mutants are less tolerant to drought (Salehin et al., 2019), and that glucosinolate breakdown products can trigger stomatal closure in Arabidopsis and *Vicia faba* (Khokon et al., 2011; Hossain et al., 2013), these results provide evidence that aliphatic glucosinolates are involved in stomatal regulation. Interestingly, glucosinolate-mediated stomatal regulation requires a functional ROS receptor kinase (GHR1; Salehin et al., 2019). Given that the myrosinase TGG1 accumulates in guard cells and is required for stomatal regulation (Zhao et al., 2008), and that glucosinolate breakdown products can regulate stomatal closure through ROS production (Khokon et al., 2011), it is conceivable that ROS link endogenous glucosinolates to stomatal regulation (Khokon et al., 2011).

Apart from growth and stomatal opening, glucosinolates may also regulate the circadian clock and flowering time. Natural presence/absence variation in the 2-oxoglutarate-dependent dioxygenase AOP2, which converts methylsulfinylalkyl glucosinolates into alkenyl glucosinolates, is linked to variation in the expression of the major flowering gene *FLC* and to variation in flowering time (Kliebenstein et al., 2001; Atwell et al., 2010). Introducing a functional AOP2 into Arabidopsis Col-0 (a natural AOP2 knockout) confirmed the flowering time effect, identified a shift in the expression of circadian genes, and showed a 1-h decrease in clock periodicity (Kerwin et al., 2011). Abolishing glucosinolate production using *Myb* transcription factor mutants led to the same periodicity shift, suggesting that the effect may be linked to the presence of the 4-MSO glucosinolate in wild-type Col-0 (Kerwin et al., 2011). The effect of the AOP locus on flowering time depends on the genetic background (Jensen et al., 2015), supporting the hypothesis that secondary metabolites are integrated into a complex and variable regulatory network. How aliphatic glucosinolates directly regulate gene expression networks and developmental

phenotypes such as flowering time remains to be tested (Burow and Halkier, 2017).

The present data suggest that glucosinolates can influence growth by multiple different mechanisms, including Target of Rapamycin regulation, auxin regulation, auxin-independent transcriptional regulation, and auxin-mediated ROS accumulation (Katz et al., 2015; Kim et al., 2015; Malinovsky et al., 2017; Salehin et al., 2019). This diversity, paired with the substantial variation in glucosinolate biosynthesis within species, creates a wealth of metabolic networks and phenotypes, which can be acted upon by natural selection. It is tempting to speculate that this diversity is a reflection of the highly diverse habitats and environments that a single species can inhabit and may provide adaptive potential beyond conserved hormonal pathways.

In addition to glucosinolates, flavonoids are implicated in regulating plant growth, development, and environmental responses. Exogenously applied flavonoids have long been known to modulate auxin transport (Stenlid, 1976). Evidence that flavonoids may also act as endogenous growth regulators came from an Arabidopsis chalcone synthase mutant, *transparent testa* (*tt4*). *tt4* plants show growth alterations that are characteristic of disturbed auxin localization, including reduced root growth and gravitropism (Brown et al., 2001). The *tt4* mutant also displays increased auxin transport (Murphy et al., 2000), which can be reversed by adding the flavonoid precursor naringenin (Brown et al., 2001). Further mechanistic studies suggest that flavonoids modulate auxin transport through several mechanisms, including interactions with auxin transporters and transport-regulating proteins (Peer and Murphy, 2007; Santelia et al., 2008). Arabidopsis roots grow away from light and flavonoids accumulate in their light-exposed sides (Silva-Navas et al., 2016). The *tt4* mutant also shows reduced light avoidance, which was linked to reduced auxin polar transport and reduced ROS accumulation, both of which can regulate cell division and elongation (Gayomba et al., 2010; Silva-Navas et al., 2016). *tt4* also displays lower accumulation of flavonols and increased ROS levels in guard cells, phenotypes associated with more rapid absisic acid–induced stomatal closure (Watkins et al., 2014). An additional link between flavonoid biosynthesis, ROS accumulation, and plant development was uncovered recently in tomato (*Solanum lycopersicum*; Muhlemann et al., 2018). The *are* mutant is defective in a flavonol 3-hydroxylase (F3H), displays reduced flavonol and increased ROS accumulation in pollen grains, and suffers from reduced pollen tube growth and integrity. The pollen tube phenotype can be rescued by the addition of antioxidants (Muhlemann et al., 2018). Flavonols are thus thought to act as antioxidants that reduce ROS accumulation and thereby regulate plant development (Hernández et al., 2009; Muhlemann et al., 2018). However, the oxidation state of a cell can directly influence signaling by altering disulfide bridge formation or other protein modifications. Thus, it is possible that flavonols also function as signals and further work is needed to differentiate between these hypotheses.

Other secondary metabolites may also regulate plant development. Diploid oat *sad2* mutants that overproduce the triterpene β-amyrin produce shorter roots and significantly more root hairs than wild-type plants, phenotypes which are absent in other mutants of the pathway that do not overproduce β-amyrin (Kemen et al., 2014). However, this phenotype cannot be phenocopied by adding β-amyrin to roots, possibly because its activity requires specific spatiotemporal accumulation patterns (Kemen et al., 2014). In *N. attenuata*, silencing a malonyltransferase that malonylates 17-Hydroxygeranylinalool diterpene glycosides reduces floral style cell size and length (Li et al., 2018b). Knocking down diterpene glycoside production by silencing a geranylgeranyl diphosphate synthase abolishes the effect of the malonyltransferase, suggesting that specific diterpene hexose decoration patterns are responsible for the flower phenotype (Li et al., 2018b). Furthermore, a labeling experiment in poplar recently uncovered that herbivore-attacked leaves can convert benzyl cyanide, a herbivore-induced volatile, to the auxin phenylacetic acid (Günther et al., 2018), thus providing a potential link between the catabolism of volatile secondary metabolites and the regulation of plant growth and development.

The examples above show how secondary metabolites can modulate growth and development through a variety of mechanisms, some of which are barely distinguishable from mechanisms normally assigned to plant hormones (Fig. 2). Whereas some of these secondary metabolite regulators are ancient and highly conserved (e.g. flavonoids, terpenes), others evolved more recently (e.g. glucosinolates and benzoxazinoids) and are restricted to specific plant families. Plants thus have both a conserved and a unique, variable, and flexible repertoire of regulators at their disposition to adjust growth and development, which likely contributes to their potential to colonize variable and challenging habitats.

Secondary Metabolites as Primary Metabolites

If secondary metabolites can regulate growth, development, and defense, can they also function as primary metabolites? Whereas primary metabolites are highly conserved, secondary metabolites evolve dynamically and are inherently variable in structure and production (Wink, 2008). This rapid evolution would seem to complicate their integration into the most fundamental workings of plant metabolism because it would require a rapid evolution of enzymes to connect these new structures into the more conserved metabolic pathways. However, evidence for secondary metabolites that are not strictly essential, but nevertheless contribute to primary metabolism, is emerging. In Arabidopsis, plants

Figure 2. Glucosinolates and benzoxazinoids as examples of secondary metabolites that blur the functional trichotomy of plant metabolism. Different functions of glucosinolates in Arabidopsis and benzoxazinoids in maize and wheat are depicted. Genes that are known to be involved in the different functions are indicated. Note that a direct role of benzoxazinoids and glucosinolates as plant primary metabolites (for instance, in the context of nitrogen/sulfur and/or energy storage) has not been clearly demonstrated so far. *MEDs and KFBs* are likely regulated by aldoxime precursors of glucosinolates. For references, see the article.
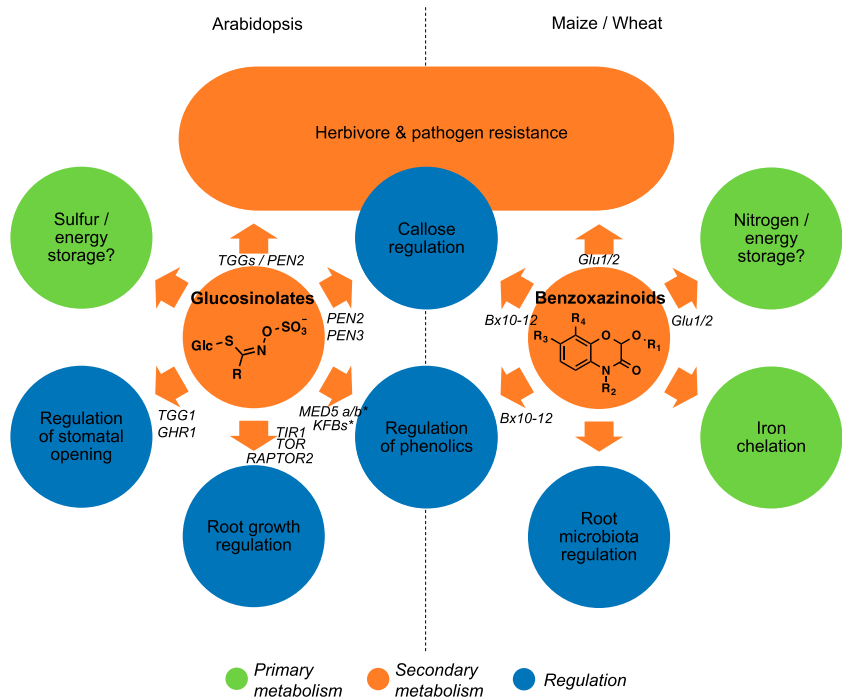
with mutations in the flavonoid pathway upstream of the FLAVANONE-3-HYDROXYLASE (F3H) show a reduction in the respiratory cofactor ubiquinone (coenzyme Q; Soubeyrand et al., 2018). Ubiquinone levels can be restored by adding dihydrokaempferol or kaempferol to the mutants. Labeling experiments demonstrate that the aromatic ring of kaempferol is integrated into ubiquinone, and that heme-dependent peroxidases likely use kaempferol to produce 4-hydroxybenzoate as a substrate for ubiquinone (Soubeyrand et al., 2018).

The integration of flavonoids into primary metabolism is perhaps not surpising, because they represent one of the oldest and most conserved classes of secondary metabolites (albeit with substantial interspecific variation in glycosylation patterns). Flavonoid evolution precedes the emergence of many innovations in plant primary metabolism, such as C_4 photosynthesis. Whether younger, more specialized secondary metabolites can act as primary metabolites is not well understood. This lack of knowledge is closely related to a limited understanding of secondary metabolite catabolism. Where do these compounds go when they are no longer needed? One would assume that reintegrating secondary metabolites into primary metabolism is beneficial for plants (Neilson et al., 2013). Such a reintegration pathway has been proposed for cyanogenic glycosides (Selmar et al., 1988). Upon deglycosylation, HCN may be assimilated into Asn via the formation of β-cyano-Ala (Selmar et al., 1988). Indeed, two sorghum (*Sorghum bicolor*) nitrilases are capable of producing Asn from β-cyano-Ala (Jenrich et al., 2007). An alternative pathway not involving the release of HCN was suggested in sorghum. In this system, nitrilases

are proposed to take the deglycosylated cyanogen and directly release ammonia and the corresponding acetate (Jenrich et al., 2007). Further support for the potential of cyanogenic glucosides as a primary metabolite store came from overexpression of a hydroxynitrile lyase, which is involved HCN formation in cassava (*Manihot esculenta*). These plants have decreased concentrations of cyanogenic glycosides and increased concentrations of total amino acids, suggesting that cyanogenic glycosides may be degraded and reintegrated into primary metabolism (Narayanan et al., 2011). The potential integration of other secondary metabolites such as glucosinolates is currently under investigation. In Arabidopsis, sulfur deficiency induces the expression of the myrosinases BGLU28 and BGLU30 (Maruyama-Nakashita et al., 2003). Under sulfur-limiting conditions, *bglu28/30* double mutants accumulate higher levels of intact aliphatic glucosinolates, contain lower amounts of Cys and protein sulfur content, and grow less than wild-type plants, suggesting that glucosinolates may serve as sulfur-storage molecules (Zhang et al., 2020).

Detailed biochemical characterization and metabolic profiling of secondary-metabolite mutants, combined with complementation and labeling experiments, are required to further corroborate the potential roles of secondary metabolites in primary metabolism and to identify additional secondary metabolites that serve as primary-metabolite precursors. Degradation of many different secondary metabolites has been observed under specific environmental conditions (Negi et al., 2014; Zipor et al., 2015). Furthermore, alterations in primary metabolites are observed in various plants

with altered secondary metabolism (Mayer et al., 2001; Narayanan et al., 2011; Huber et al., 2016; Machado et al., 2017; Zhang et al., 2020), and the accumulation of specific secondary metabolites has been associated with storage and growth in microevolutionary studies (Heath et al., 2014). Keeping an open mind about the capacity of plants to evolve integrated metabolic networks is warranted to gain a better comprehension of the prevalence and importance of secondary metabolites as precursors of primary metabolites.

Secondary Metabolites as Facilitators of Micronutrient Uptake

An additional example that further blurs the distinction between primary and secondary metabolism is plant micronutrient uptake. Grasses excrete low M_r compounds into the rhizosphere to chelate micronutrients such as iron and thus make them biologically available (Curie and Briat, 2003). Recent work suggests that secondary metabolites are likely important for iron uptake in both herbs and grasses. Chemical removal of excreted phenolic acids from the nutrient solution of red clover (*Trifolium pretense*) was found to result in iron deficiency in red clover (Jin et al., 2007). Subsequently, an Arabidopsis mutant, which is deficient in the 2-oxoglutarate-dependent dioxygenase Feruloyl-CoA 6′-Hydroxylase 1 and thus no longer able to produce coumarins, was found to suffer from iron deficiency under alkaline conditions (Schmid et al., 2014). Similarly, young maize benzoxazinoid mutants that do no longer produce and excrete benzoxazinoids were found to suffer from iron deficiency when growing in the presence of iron salts (Hu et al., 2018). Both benzoxazinoids and coumarins are able to chelate iron in vitro (Bigler et al., 1996; Mladenka et al., 2010). Because these complexes are essential for plant growth and development by providing essential micronutrients, they should, according to definition, be classified as primary metabolites, thus providing another illustration of how secondary metabolites can turn into primary metabolites under given conditions.

ADAPTIVE EXPLANATIONS FOR METABOLIC INTEGRATION OF SECONDARY METABOLITES

There is now ample evidence for secondary metabolites that are regulators and precursors of primary metabolites. But why would plants evolve an integrated metabolism in which the same metabolite class has multiple functions that incorporate growth, development, defense, and regulation? Plants have large, interconnected metabolic networks at their disposition. Natural selection acts on these metabolic networks, resulting in the evolution of network topologies that maximize fitness. Over evolutionary time, these topologies likely include dynamic transitions

between secondary metabolites and hormones, for instance (Malinovsky et al., 2017; Sun et al., 2019b). Overall, the functional integration of secondary metabolites at a given point in evolution is a likely consequence of the interaction between complex environments with highly connected plant metabolic networks. Below, we discuss the potential benefits of plant secondary metabolite metabolic integration that may have favored their use as regulators and primary metabolites.

Plant Secondary Metabolites as Reliable Readouts of Defense Activation

Plants control defense activation to save metabolic energy and avoid self-damage. Defense investment is typically titrated through feedback regulation, including both positive and negative feedback loops that are built into early defense signaling (Hu et al., 2015; Li et al., 2015) and hormonal networks (Gilardoni et al., 2011; Liu et al., 2019). A limitation of these feedback loops is that they do not provide direct information about the final level of defense activation (i.e. the production of defense metabolites per se). Because herbivores and pathogens may interfere with the production of defense compounds at many levels, including in the final steps of biosynthesis (Jones et al., 2019), integrating them directly into regulatory feedback loops may allow plants to more accurately monitor and adjust defense accumulation. Using secondary metabolites as defense activation readouts may also help plants to optimize synergies between different defenses and to compensate for accidental failures of specific defense pathways. The increasing number of examples showing that plant secondary metabolites regulate defenses (see section "Secondary Metabolites as Regulators of Plant Defense") hint at the existence of such systems.

As many secondary metabolites are compartmentalized and/or stored in inactive forms, their decompartmentalization and/or activation likely also helps plants to recognize tissue damage and other forms of environmental stress. In this case, the metabolites would be used as damage–associated molecular patterns (DAMPs). Green-leaf volatiles are an example of secondary metabolites that are also DAMPs (Tanaka et al., 2014; Quintana-Rodriguez et al., 2018). Another potential example of secondary metabolites as DAMPs is the previously discussed links between indolic glucosinolates and DIMBOA regulation of callose upon pathogen attack. Interestingly in this case, the secondary metabolite/DAMPs are linked to endogenous responses to pathogen-associated molecular patterns (e.g. FLS2; Clay et al., 2009) and stomatal closure upon drought stress (Salehin et al., 2019).

Given these considerations, secondary metabolites may be common readouts of defense activation and damage may have favored their evolution as defense regulators.
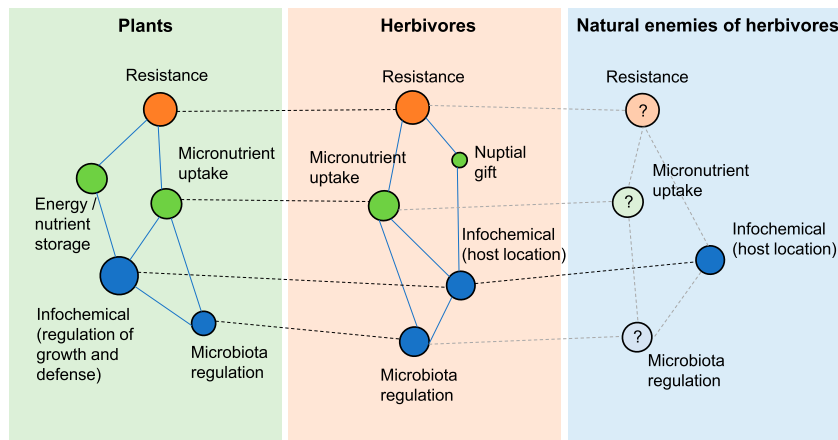
Figure 3. Functional integration of plant secondary metabolites shapes plant–herbivore and tritrophic interactions. Schematic representation of how different functions of secondary metabolites are used by plants, herbivores, and natural enemies of herbivores is shown. Plants use secondary metabolites for multiple purposes, including resistance, regulation, and primary metabolism (see Fig. 2). Recent work suggests that this multifunctionality is mirrored in adapted herbivores, which also use secondary metabolites for multiple purposes, including similar and new functions. Little is known about how adapted natural enemies use secondary metabolites, but multifunctional integration across three trophic levels is likely (Box 2). Circles represent hypothetical individual secondary metabolites (for color code, refer to Figs. 1 and 2). Solid lines indicate metabolic connections within an organism. Dashed lines indicate similar functions of the same compounds in different organisms.

Metabolic Network Specialization as a Potential Means to Resist Manipulation

Herbivores, pathogens, and viruses can interfere with defense hormone signaling and thereby manipulate plants for their own benefit (Kazan and Lyons, 2014; Stahl et al., 2018). The high degree of conservation in defense hormone signaling may in fact favor the evolution of biotic manipulation of plant signaling (Berens et al., 2017). For example, if an attacking organism evolves the ability to alter jasmonate signaling, this may provide it a fitness benefit on a wide variety of host plants and may reduce the advantage for plants to evolve new inducible resistance mechanisms regulated by these hormones. One possibility to solve this problem would be to use less-conserved metabolites as defense regulators. If a plant had the ability to use these metabolites, it would be less likely to fall prey to host switching by hormone-manipulating enemies. The evolution of (specialized) secondary metabolites into regulatory networks may thus be promoted through the evolution of manipulation strategies in plant enemies. Clear examples supporting this hypothesis are currently lacking. As the biosynthesis of defense-regulating secondary metabolites such as glucosinolates is at least partially controlled by conserved phytohormonal pathways (Schweizer et al., 2013), plant enemies that are capable of overcoming these conserved pathways may also suppress more specific regulators. Interestingly, an opposite pattern has also been found for the tomato leaf spot fungus, which uses a hydrolase to detoxify steroidal glycoalkaloids and benefits from the defense-suppressing properties of the resulting breakdown products (Bouarab et al., 2002). This illustrates that specialized plant enemies may also misuse the regulatory properties of secondary metabolites of their host plants.

Multifunctionality as a Cost-Saving Strategy

Producing secondary metabolites has energetic and metabolic costs (Gershenzon, 1994). These costs are not always evident (Züst et al., 2011; Machado et al., 2017), and may mostly occur under specific environmental conditions such as strong competition and nutrient limitation (Cipollini et al., 2018). Plants likely manage costs of secondary metabolite production through the regulation of biosynthesis, but controlled recycling of the resulting compounds would enhance the plants ability to recoup costs in challenging environments (Neilson et al., 2013). Secondary metabolites that are induced upon environmental stress could for instance be recycled back into primary metabolism once the stress subsides. One way of testing this hypothesis is to manipulate secondary metabolite recycling by targeting enzymes involved in their degradation, such as glucosidases (Morant et al., 2008) or nitrilases (Jenrich et al., 2007). With use of this approach, a link between the degradation of cyanogenic glycosides and plant protein supply was uncovered (Narayanan et al., 2011), supporting the hypothesis that reintegration of secondary compounds into primary metabolism may be advantageous for the plant. A caveat of this approach is that it remains difficult to disentangle a direct contribution of the generated catabolites to primary metabolism from their potential regulatory roles. A more detailed understanding of secondary metabolite signaling and catabolism would help to explore the role of secondary metabolite reintegration as a cost-saving strategy.

Another way to minimize costs is to use the same secondary compound for multiple purposes (Neilson et al., 2013). As many secondary compounds are

BOX 1. Case study of secondary metabolite multifunctionality

Young maize plants produce high amounts of benzoxazinoid secondary metabolites. Benzoxazinoids are used by the plant for multiple purposes, including direct resistance against herbivores (Glauser et al., 2011; Maag et al., 2016), the regulation of callose deposition as a defense against aphids (Ahmad et al., 2011; Meihls et al., 2013), and iron uptake through their capacity to chelate iron in the rhizosphere (Hu et al., 2018). Recent work shows that the western corn rootworm, a highly specialized maize pest, phenocopied these functions and is able to use benzoxazinoids for multiple purposes as well. The larvae of the herbivore use benzoxazinoids as foraging cues in the rhizosphere (Robert et al., 2012), are able to extract iron from benzoxazinoid-iron complexes to maximize their own growth (Hu et al., 2018), and are able to store benzoxazinoids to resist their own enemies, namely entomopathogenic nematodes (Robert et al., 2017). Strikingly, both the plant and the herbivore have evolved to modify the structure, localization, and stress-induced modification of benzoxazinoids to fit these different functions (Ahmad et al., 2011; Maag et al., 2016; Robert et al., 2017). This example illustrates how a detailed understanding of the functional integration of plant secondary metabolites can help to unravel the chemical underpinning of plant–herbivore interactions.

Box 1. Case study of secondary metabolite multifunctionality. Cited articles: Glauser et al., 2011; Robert et al., 2012, 2017; Maag et al., 2016.

BOX 2. Multi-functionality of plant secondary metabolites in tritrophic interactions

Beyond plants and herbivores, the multi-functionality of plant secondary metabolites may also extend to the third trophic level. The current view on this topic is that i) plant primary metabolites influence tritrophic interactions by directly or indirectly feeding natural enemies of herbivores (Hunter, 2003; Sarfraz et al., 2009; Ugine et al., 2019), ii) volatile secondary metabolites can serve as foraging cues (Aartsma et al., 2017; Turlings and Erb, 2018), and iii) non-volatile secondary metabolites can reduce herbivore host quality and thereby negatively affect natural enemies of herbivores, in particular when they are sequestered by specialized herbivores (Nishida, 2002; Opitz and Müller, 2009). To what extent natural enemies of herbivores may use plant secondary metabolites as primary metabolites for their own nutrition or as hormone-like regulators is currently unknown. Natural enemies of herbivores can metabolize secondary metabolites (Sloggett and Davis, 2010; Robert et al., 2017; Sun et al., 2019a), and can evolve resistance towards their toxic effects (Fink and Brower, 1981; Rafter et al., 2017; Zhang et al., 2019). Understanding if and how plant secondary metabolites are integrated into the metabolism of herbivore natural enemies represents an important current frontier in chemical ecology. This field can benefit from current insights into the blurred functional trichotomy of plant metabolism.

Box 2. Multifunctionality of plant secondary metabolites in tritrophic interactions. Cited articles: Fink and Brower, 1981; Hunter, 2003; Sarfraz et al., 2009; Sloggett and Davis, 2010; Aartsma et al., 2017; Rafter et al., 2017; Robert et al., 2017; Turlings and Erb, 2018; Sun et al., 2019a; Ugine et al., 2019; Zhang et al., 2019.

chemically reactive, they need to be managed by the plant through (potentially costly) storage, inactivation, and/or resistance mechanisms, including specialized cells, ducts, and glands (Sirikantaramas et al., 2008). By employing the same compound class for multiple purposes, plants may spread these fixed costs across more fitness components and increase their competitiveness. Metabolic costs may also be lowered by using the same biosynthetic machinery to produce different compounds for different purposes. Whereas the cost-saving aspects of multifunctionality are difficult to quantify, multifunctionality seems to be a widespread property of secondary metabolites, as discussed above, and it is difficult for this multifunctionality to evolve without benefit.

ECOLOGICAL CONSEQUENCES OF THE METABOLIC INTEGRATION OF SECONDARY METABOLITES

The separation of low M_r compounds into primary metabolites, secondary metabolites, and hormones has shaped our ecological and evolutionary thinking of plant–environment interactions. If we abolish this view in favor of a more integrated perspective (i.e. where secondary metabolites can have regulatory roles and can provide precursors for primary metabolites), we

can derive new hypotheses on plant defense patterns and plant–herbivore interactions. These hypotheses are likely to improve our understanding of the ecological roles of plant secondary metabolites in the future.

Ontogenetic Patterns of Secondary Metabolite Production

Many secondary metabolites show distinct ontogenetic accumulation patterns, with concentrations varying over time and between tissues. Ecological theory explains this within-plant variation using resource constraints, allocation costs, and variation in herbivore pressure (McKey, 1974; van Dam, 2009; Meldau et al., 2012; Schuman and Baldwin, 2016; Barton and Boege, 2017). The above theories are all based on costs and benefit relationship, with the benefit typically being limited to herbivore resistance. Given the blurred trichotomy of plant secondary metabolism, the ecological balance sheet may be improved by taking into account multifunctionality (Barton and Boege, 2017). A drop in secondary metabolite levels, as is often observed a few weeks after germination or at the onset of flowering, for instance (Meldau et al., 2012; Barton and Boege, 2017), may reflect an increased need of primary metabolites and nutrients rather than a drop in herbivore pressure. Similarly, strong expression of secondary metabolites in roots may not only be the result of high tissue value and a high risk of root herbivore attack, but may simply reflect additional functions of the compounds such as micronutrient uptake and microbial conditioning (Hu et al., 2018; Stringlis et al., 2018). Our understanding of ontogenetic allocation patterns of secondary metabolites may thus improve if we take their full metabolic integration and potential multifunctionality into account and do not limit their considered benefits to herbivore resistance.

Defense Metabolites in Plant–Herbivore Interactions

The functional trichotomy used to define plant metabolites has also shaped our understanding of how these metabolites influence plant–herbivores interactions. Herbivores are assumed to forage for primary metabolites while trying to avoid the negative effects of secondary metabolites through behavioral and metabolic adaptations (Behmer, 2009; Stahl et al., 2018). If we accept that secondary metabolites can also be regulators and precursors of primary metabolites, then it becomes conceivable that they may have similar roles in herbivores. The root-feeding larvae of the western corn rootworm for instance forage for iron-benzoxazinoid complexes to acquire iron and improve their growth, thus effectively using a plant secondary metabolite as a primary metabolite (Hu et al., 2018). Several other herbivores also gain more weight in the presence of plant secondary metabolites (Meldau et al., 2009; Richards et al., 2012; Marti et al., 2013; Veyrat et al., 2016; Wetzel et al., 2016), and it is conceivable that some of these effects may be due to the capacity of the herbivores to metabolize these compounds. Recent examples also hint at the possibility that plant secondary metabolites may have hormonal functions in herbivores. In rice, knocking down *CYP71A1*, a gene responsible for the production of serotonin, a monoamine neurotransmitter, reduces the performance of the rice brown planthopper (*Nilaparvata lugens*). Adding serotonin to an artificial diet enhances its performance (Lu et al., 2018), suggesting that the herbivore may benefit from the hormonal properties of this plant metabolite. Plants may also benefit from producing secondary metabolites that act as (de)-regulators of herbivore physiology. Spinach (*Spinacia oleracea*), for instance, produces the molting hormone 20-hydroxyecdysone (Bakrim et al., 2008), which can interfere with caterpillar development (Kubo et al., 1983).

In general terms, a plant's metabolism is shaped by a dynamic landscape of environmental selection pressure; conversely, the metabolic network of herbivores is shaped by the functional and chemical potential of plant metabolites within the herbivore's own selection landscape. One can thus expect that, similar to what Rhoades postulated for plants (Rhoades, 1977), any chemical system taken up by a herbivore must necessarily be integrated into its total metabolic scheme, and multiple functions of plant secondary metabolites are to

OUTSTANDING QUESTIONS

- Do secondary metabolites perform regulatory functions by binding to receptor proteins, by altering specific non-receptor proteins, or through general autotoxicity/reactivity mechanisms?
- Do secondary metabolites that regulate multiple plant processes act through single signaling pathways or do they have multiple direct targets?
- How are secondary metabolites re-integrated into primary metabolism?
- Is multifunctionality and metabolic integration common to major secondary metabolite classes?
- Can plants protect themselves from enemies that manipulate canonical signaling pathways by evolving specialized secondary metabolite signaling?
- How common is the misuse of plant secondary metabolites as primary metabolites and regulators by adapted herbivores?
- Can natural enemies use plant secondary metabolites as primary metabolites and physiological regulators?

be expected, some of which likely mirror their multiple functions in plants (Fig. 3). Specialist herbivores are known to use secondary metabolites as infochemicals (e.g. foraging cues), and some also sequester defenses to protect themselves against herbivore natural enemies (Nishida, 2002; Opitz and Müller, 2009), in analogy to the use of these chemicals as defense regulators and resistance factors in plants (Fig. 3). Cabbage aphids (*Brevicoryne brassicae*) are an illustrative example in this context, as they can activate glucosinolates by producing their own myrosinases (Bridges et al., 2002; Kazana et al., 2007). This allows them to use glucosinolates as two-component defense system against predators (Kazana et al., 2007). As glucosinolate breakdown products (isothiocyanates) also increase aphid responses to alarm pheromones (Dawson et al., 1987), it was proposed that aphid-released isothiocyanates may also act as danger signals (Bridges et al., 2002). Another example where herbivores use secondary metabolites for several purposes that mirror their multiple uses by plants are again benzoxazinoids, which are used as defense metabolites and siderophores by a specialist root herbivore in maize (Box 1). Apart from mirroring plant functions, adapted herbivores can also use plant secondary metabolites for herbivore-specific functions. Cyanogenic glycosides, for instance, can be used by specialized lepidoptera as defenses and nuptial gifts (Zagrobelny et al., 2018), and glucosinolates are part of the pheromone blend of flea beetles (*Phyllotreta striolata*; Beran et al., 2016).

These examples illustrate that, as in plants, secondary metabolites can act as defenses, regulators, and precursors of primary metabolites in herbivores. Furthermore, the multifunctionality of plant secondary metabolites for plants is reflected by the multifunctional misuse of these compounds by specialized plant-feeders (Fig. 3). Whether similar phenomena can also be observed in natural enemies of herbivores, thus also shaping tritrophic interactions, is an exciting open question (Box 2).

In summary, the field of plant–herbivore interactions is likely to benefit from abandoning functional preconceptions of plant secondary metabolites and to focus on a better understanding of the metabolic integration of plants and insects through untinged glasses. Comparative metabolomics of plant and herbivore tissues (Jansen et al., 2009) and parallel genome-wide screens of plants and herbivores (Nallu et al., 2018) are promising approaches to assess plant–herbivore interaction and to identify metabolite functions and effects in herbivores without prior functional assumptions.

CONCLUDING REMARKS

The functional separation of plant-derived, low M_r organic compounds into primary metabolites, secondary metabolites, and hormones has proven to be a useful approximation over the last decades. However, recent work has shown that several classes of plant secondary metabolites are highly integrated into plant metabolism and can serve as both regulators and primary metabolites. Thus, it is likely that most secondary metabolites have additional functions for plants. Taking into account these additional functions (see Outstanding Questions), we can refine key concepts in plant-environment interactions and improve our understanding of the chemical ecology of plants and their enemies.

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