Adaptive mechanisms of plant specialized metabolism: connecting chemistry to function

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Abstract

As sessile organisms, plants evolved elaborate metabolic systems that produce a plethora of specialized metabolites as a means to survive challenging terrestrial environments. Decades of research has revealed the genetic and biochemical basis for a multitude of plant specialized metabolic pathways. Nevertheless, our knowledge is still limited concerning the selective advantages provided by individual and collective specialized metabolites to the reproductive success of diverse host plants. Here, we review the biological functions conferred by various classes of plant specialized metabolites in the context of plants' interaction with their surrounding environment. To achieve optimal multifunctionality of diverse specialized metabolic processes, plants employ various adaptive mechanisms at subcellular, cellular, tissue, organ and interspecies levels. Understanding these mechanisms and the evolutionary trajectories underlying their occurrences in nature will ultimately enable efficient bioengineering of desirable metabolic traits in chassis organisms.

Introduction

Approximately 470 million years ago, pioneering plants migrated from water to land, and have since flourished, establishing the foundation of terrestrial ecosystems as we know them now[1]. Unlike animals that leverage highly complex motor and nervous systems to actively hunt for food, evade danger and find mates, plants are sessile organisms that have to cope with all challenges arising from a fixed location throughout their entire life cycle. As a result, plants evolved a plethora of functionally diverse metabolites as a main evolutionary strategy to enhance their reproductive success. The great expansion of specialized metabolic networks thus enabled plants to diversify, occupy multitude terrestrial environmental niches, and establish intricate biotic interactions with other co-evolving organisms.

Humans have consumed plants for their nutritional and medicinal properties for millennia[2]. Modern scientific inquiry into the chemical makeup of plants began in the early 19th century, which gave birth to the field of phytochemistry (**Figure 1**). Since the first isolation of the antimalarial drug quinine from the bark of the cinchona tree in the 1820s, hundreds of thousands of natural products have been discovered from a wide selection of plants, and in fact, most early modern pharmaceuticals are plant natural products or their derived analogs[3]. The advancement of phytochemistry as a field also promoted the development of analytical chemistry, and later organic chemistry, laying the foundation for modern chemical and pharmaceutical industries. Studies of plant natural product biochemistry began in the early 20th century. Using radiotracing and basic enzymology, knowledge regarding how diverse classes of plant specialized compounds are derived from various primary metabolite precursors was uncovered for the first time. The rise of model organisms (e.g. *Arabidopsis thaliana* and *Oryza sativa*) in the 1990s, together with the wide adoption of molecular genetics and recombinant DNA technologies, greatly facilitated plant biochemistry research and contributed to the elucidation of the molecular basis for numerous important plant specialized metabolic pathways, ranging from relatively conserved networks such as phenylpropanoid metabolism and phytohormone biosynthesis to more taxonomically restricted pathways such as glucosinolate biosynthesis. Entering the 21st century, the advent of next-generation sequencing technologies and burgeoning synthetic biology tools further

ignited a renaissance of phytochemistry research, which allows researchers to return to diverse non-model plants for exploration of their vast natural product biosynthetic pathways[4,5].

While the lion's share of plant specialized metabolism research has been devoted to elucidating unknown natural product biosynthetic pathways resulting in considerable advances in this area, much less is known about the biological functions of these specialized metabolites in their native plant hosts under varying environmental conditions. Moreover, it is well recognized that beyond the emergence of new catalysts during plant metabolic evolution, subcellular-, cellular-, tissue-, organ- or interspecies-level adaptations have also occurred in diverse plants tailoring to the functions of various specialized metabolic traits. In this review, we focus on recent literature that addresses the "form and function" question of plant specialized metabolism. These underexplored aspects of plant specialized metabolism are essential for an integral understanding of the role of metabolism in plant organismal evolution, and for devising efficient metabolic engineering strategies for producing high-value plant natural products.

The roles of plant specialized metabolites in establishing dynamic below-ground interactions

The world of plant specialized metabolites is enormous with more than 200,000 different compounds known to date and more to be discovered[6]. An increasing body of evidence indicates that plants utilize these diverse compounds to manipulate their surroundings via plant-plant, plant-insect and plant-microbe interactions. Metabolites may be produced individually, or in mixtures, in response to certain environmental or developmental cues and serve as signaling molecules, attractants, repellents or inhibitors of other organisms[7]. Certain metabolites carry information about the physiological and metabolic status of the host plant that is readily interpreted by other plants, insects, and microbes, which respond accordingly[8]. However, our ability to decrypt the chemical languages of plants is still in its infancy.

The roles of plant specialized metabolites in above-ground communications have been extensively studied and reviewed[9]. Recent advent of metagenomics and

untargeted metabolomics techniques, however, greatly facilitated research in plants' below-ground interspecies and interkingdom interactions[10]. Plant metabolites released into the rhizosphere via root exudation can (i) serve as allelochemicals inhibiting the growth of neighboring plants[11], (ii) drive plant-microbiome interactions, (iii) function as defense compounds against soil-borne microbial pathogens, and (iv) shape the composition of the root microbiota (**Figure 2a**). Flavonoids, for example, contribute to all above functions, as they are involved in allelopathic interference, repel parasitic nematodes, inhibit pathogenic fungi, and attract mycorrhizal fungi that form beneficial symbiosis with plants[12]. In addition, the makeup of the Arabidopsis root bacterial community can be modulated by iron-mobilizing coumarins that inhibit the proliferation of *Pseudomonas* species via a redox-mediated mechanism, as was discovered using a combination of a synthetic community of Arabidopsis root-isolated bacteria and mutants deficient in various specialized metabolic pathways[13]. At the same time, plant roots are constantly exposed to thousands of different microbes, and the rhizosphere microbiota promote systemic changes in the metabolite profile of root exudates[14]. Thus, not only do plant root exudates shape root-associated microbiota, but also the rhizosphere microbiome modulates the chemical composition of root exudation. For example, in tomato, glycosylated azelaic acid was recently identified as a potential microbiome-induced signaling molecule, which is transported via shoots to uncolonized roots and is exuded in the free acid form to promote rhizosphere chemical diversification and interactions within microbial community[14]. Plants can also further metabolize signal molecules released from soil organisms, converting these metabolites for their own benefits, such as ascaroside pheromones secreted by parasitic nematodes, to nematode deterrent as a means to reduce infection[15] (**Figure 2b**).

Beyond below-ground effects, root exudates can carry information to receiver plants that affects their above-ground performance. Fitness of the next generation of plants, for example, can be influenced by altered soil microbiota as a result of exudation of bioactive secondary metabolites from the parents' roots. Indeed, maize roots secrete to the rhizosphere substantial amounts of tryptophan-derived benzoxazinoids. In addition to their allelopathic functions[11], they trigger changes in microbiome composition, which in turn increases jasmonic acid signaling in leaves of the next

generation of plants, leading to enhancement of jasmonic acid-dependent defense and herbivore resistance while decreasing plant growth[16]. These observed phenotypic changes were attributed to 6-methoxy-benzoxazolin-2-one (MBOA), a compound produced by degradation of the maize-secreted benzoxazinoids[16] (**Figure 2c**).

Communication via root exudates extends beyond defense, and can carry information about other environmental conditions as well. For instance, application of root exudates from *Brassica rapa* grown in long-day conditions can promote accelerated flowering of plants grown under short-day conditions[17]. Similarly, an informational cascade about water deficiency can be passed from drought-stressed plants to their neighbors, which in turn propagate this information further to plants that were never in contact with original stressed individuals. To date, the nature of the below-ground signaling compounds in these plant-plant interactions is still unknown and awaits further investigation.

Volatiles released from roots to the soil not only act in below-ground plant defense, but also influence ecological interactions between herbivores and neighbouring plants[18]. It is well known that (E)-β-caryophyllene released by insect-damaged maize roots recruits entomopathogenic nematodes, thus protecting plants via below-ground tritrophic interaction[19]. However, more recently it was shown that a mixture of sesquiterpenes with a high abundance of (E)-β-caryophyllene constitutively released from the spotted knapweed (*Centaurea stoebe*) roots can modify plant-herbivore interactions of different sympatric plant species by increasing their susceptibility to herbivores[20] (**Figure 2d**). In addition to their influence on herbivory, the same mixture of volatiles can increase germination and growth of neighboring plant species, thus contributing to below-ground plant-plant interactions and impacting the structure of natural plant communities[21]. However, what benefit the emitters attain from promoting the growth and/or herbivory of competitors remains unknown.

Several recent studies also demonstrated that insects and other phytoparasites can exploit plants' metabolic status for their own benefits. By manipulating the enzymatic machinery of the turpentine tree (*Pistacia palaestina*), gall-inhabitting aphids enhance monoterpene accumulation thus intensifying their own defences against natural enemies[22]. Host plant manipulation by insects can also be extended to

neighboring plants via airborne signals. Indeed, attacking whiteflies on tomato plants induce release of volatiles that reduce levels of jasmonic acid-mediated defense compounds in neighboring plants, making the latter more susceptible to infestation[23]. During plant colonization, some nematodes and biotrophic fungi secret chorismate mutase, thereby suppressing plant immunity via perturbing salicylic acid biosynthesis[24,25].

Linking specialized metabolism to plant body plan adaptation

Metabolic evolution has profoundly impacted body plan adaptation in many plants, contributing to the development of highly specialized cell and tissue types that enable specialized metabolite production and/or storage. Prominent examples include glandular trichomes[26], latex-producing laticifers[27], pigment-accumulating petal epidermal cells[28], and root tubers[29], some of which are discussed below. Moreover, numerous specialized metabolic pathways are interconnected with phytohormone biosynthetic pathways, or produce specific downstream metabolites that have gained new signaling properties, in turn regulating plant growth and development in coordination with overall metabolic function.

Trichomes are hair-like epidermal protuberances found in almost all plants, and serve as the first line of physical defense for the host plant against herbivory[30]. In about 30% of all vascular plants, simple ancestral trichomes have evolved into structurally more elaborate glandular trichomes which produce and store a wide array of specialized metabolites to instigate an extra layer of chemical defense[26]. In-depth microscopic examination of cannabis (*Cannabis sativa* L.) unveiled three morphologically distinct types of glandular trichomes on female flowers, classified as stalked, sessile, and bulbous trichomes[31]. Among these, stalked glandular trichomes contain the highest cannabinoid levels[31] (**Figure 3a**). The stem portion of the stalked glandular trichome is photosynthetic and supplies nutrients to the nonphotosynthetic head. Secretory disc cells situated at the base of the head manufacture cannabinoids as well as other terpenes and transport them into the balloon-like secretory cavities at the top[31]. Analysis of the glandular-trichome-specific transcriptome uncovered a rich set of candidate genes likely involved in cannabinoid metabolism and trafficking[31]. However, the exact

molecular machineries underlying these highly specialized and coordinated cellular processes are yet to be fully characterized. Like cannabis, nightshade plants also harbor several morphological types of glandular trichomes within individual species[32]. Instead of cannabinoids, the secretory glandular trichomes of nightshade plants produce structurally diverse acylsugars as lineage-specific defense compounds against herbivores and pathogens[32]. Cell-type-specific metabolomic analysis revealed distinct specialized metabolite profiles among different glandular trichome types in tomato, suggesting divergent biological functions associated with various glandular trichome types[32]. Furthermore, genetic variations of several acylsugar biosynthetic genes found in cultivated and wild tomato species, including acylsugar acyltransferases and acylsucrose fructofuranosidases, contribute to the rapid expansion of acylsugar diversity within the *Solanum* genus[33]. In addition to the direct anti-herbivory properties of acylsugars, research of coyote tobacco (*Nicotiana attenuata*) in its native ecological environment uncovered an intricate indirect protective mechanism: ingestion of acylsugars by larvae of Lepidopteran herbivores imparts a distinct volatile profile to their body, tagging them for predation by the rough harvester ants (*Pogonomyrmex rugosus*)[34].

An estimated 20,000 plants, across 40 families, have evolved laticifers, which are specialized elongated secretory cells found in leaves and stems that contain a white viscous material known as latex[27] (**Figure 3b**). Laticifers can either be individual long coenocytic cells (non-articulated), which are among the longest cells known in plants, or be composed of multiple cells separated by their respective cell walls (articulated)[27]. The main components in latex by dry weight are long-chain polyisoprene units (e.g. natural rubber)[27], while a broad range of specialized metabolites are also present, such as terpenoids, alkaloids, dihydroxybenzoic acids, furanocoumarins, and flavonoids among others[35]. Upon physical damage, latex rapidly oozes out from the broken laticifers at the wound site, and plays dual roles in chemical defense against insects and other herbivores and wound healing through coagulation[36]. A well studied example is the latex of opium poppy (*Papaver somniferum*) which contains morphine, a benzylisoquinoline alkaloid well known for its potent agonist activity against mammalian opioid receptors[37]. Triterpenoid cardenolides and sesquiterpene lactones found in the

latex of milkweed (*Asclepias spp.*) and common dandelion (*Taraxacum officinale*), respectively, have been shown to act as effective defense molecules against various herbivores[38,39]. In addition to specialized metabolites, latex also contains an array of defence proteins such as serine and cysteine peptidases, chitinases, thaumatin-like protein and others, with comprehensive roles in defense against herbivory[40]. Although latex-producing laticifers provide a strong line of defence against most herbivores, some insects, especially those that specialize in feeding on specific host plants, have developed strategies to circumvent ingesting latex, illustrating the evolutionary arms race between plant-eating insects and host plants. Two such specialists, the caterpillars of the monarch butterfly *Danaus plexippus* and the arctiids *Pygarctia roseicapitis*, cut veins on their host plant's petiole in a behavior called trenching[41,42], to allow the latex to leak from the wound and then feed on the distal, latex-deficient portion of the leaf.

The burgeoning specialized metabolic pathways sometimes stem from branches of primary metabolism that also support phytohormone biosynthesis. Such metabolic links thus impose control over plant growth in response to certain specialized metabolic states[43] (**Figure 3c**). For example, cytosolic phenylalanine biosynthesis, which is necessary for optimal production of many phenylpropanoid natural products, influences tryptophan-dependent auxin production via a shared phenylpyruvate intermediate[44]. Similarly, auxin biosynthesis and signaling are also tied to indole glucosinolates, a class of defense compounds found in Brassicaceae plants. Not only do the major plant auxin indole-3-acetic acid (IAA) and indole glucosinolates share the common precursor tryptophan, but indole glucosinolate biosynthetic intermediates also suppress metabolic flux towards phenylpropanoids through mediator- and proteome-dependent degradation of phenylalanine ammonia-lyase (PAL), the first committed enzyme of phenylpropanoid metabolism[45]. This complex regulatory network therefore enables the coordination of auxin-mediated growth control with multiple defense compound biosynthetic pathways.

An increasing number of specialized metabolites have been recognized to contain recently evolved signaling properties. For example, indole glucosinolate breakdown product indole-3-carbinol acts as an auxin antagonist and induces indole-3-carbinol-dependent autophagy in Arabidopsis root upon wounding[46,47]. Another aliphatic glucosinolate, namely 3-hydroxypropylglucosinolate (3OHP), inhibits root growth and

development in Arabidopsis through the Target of Rapamycin (TOR) signaling pathway[48] (**Figure 3d**). Terpene metabolism has also yielded numerous niche signaling molecules. For instance, an ancestral catabolite of the plant stress hormone abscisic acid (ABA), phaseic acid, was recruited by seed plants to function as a biased ligand that only activates a subset of the ABA receptor family proteins to provide nuanced regulation of seed germination and long-term drought response[49]. Additionally, other carotenoid-derived terpenoids, including anchorene, zaxinone, and retinal, were recently reported to impact various aspects of root development either through modulating classical phytohormone signaling pathways[50,51] or by engaging novel ligand-activated signaling pathways[52]. In petunia flower, sesquiterpenes released by the tubes within the enclosed floral buds accumulate in the pistils and are required for optimal pistil growth, as this inter-organ aerial transport likely coordinates the timing of pistil maturation with petal development to ensure successful reproduction[53]. Similarly, in *Nicotiana attenuata* flower, malonylated 17-hydroxygeranylinalool diterpene glucosides regulate floral style length by influencing stylar cell size[54]. These observations suggest that the rise of new signaling properties among specialized metabolites might be a common phenomenon during plant evolution, and further implicate the presence of respective specialized signaling pathways waiting to be uncovered.

Subcellular and intercellular mechanisms that enhance the multifunctionality of specialized metabolism

To achieve optimal multifunctionality of a given specialized metabolic pathway, such as high metabolic output, alleviation of enzyme inhibition by structurally similar metabolites[55], or avoidance of autotoxicity[56], many plant specialized metabolic processes partition into different subcellular compartments or sometimes across different cell types, and rely on transport of intermediates to bring spatially separated enzymatic steps into a complete biosynthetic pathway. The infamous pungent-tasting glucosinolates of the cabbage family, their metabolism, transport, and the release of the ‘mustard bomb’ are an exemplary case illustrating many aspects of these subcellular and intercellular mechanisms, which have been extensively reviewed by others[57]. Here,

we focus on recent literature that provide new insights on higher-order organization of numerous plant metabolic systems.

Various classical eukaryotic organelles are common sites of compartmentalized plant specialized metabolic processes. For instance, phenylpropanoid biosynthesis occurs mainly in the cytosol but relies predominantly on plastidial production of the phenylalanine precursor (**Figure 4a**)[58]. As an integral part of this metabolic network, the recently discovered plastidial cationic aromatic amino acid transporter (CAT) was shown to control flux through the network, influence organellar metabolite concentrations, and relax naturally occurring feedback regulation of phenylalanine biosynthesis in plastids[58]. On the other hand, a drastic increase in phenylalanine levels in cytosol is tempered by sequestering it from the metabolically active pool into the vacuole via action of a vacuolar CAT-family transporter, thereby sustaining cytosolic homeostasis and preventing toxicity[59].

Sequestration of specialized metabolites in the vacuole is a common strategy used by plants to accumulate high quantities of specialized metabolites[60]. Nevertheless, vacuoles can also host certain enzymatic steps of natural product biosynthesis. In *Catharanthus roseus,* the vacuolar strictosidine synthase conjugates tryptamine and secologanin imported from the cytosol to form strictosidine, which is subsequently exported by tonoplast-localized nitrate/peptide family transporter en route to formation of monoterpene indole glucoside final products[61]. Moreover, vacuole-localized papain-like cysteine proteases and asparaginyl endopeptidases are responsible for several proteolytic cleavage steps in the biosynthesis of various classes of plant ribosomally synthesized and post-translationally modified peptide (RiPP) natural products[62,63].

In addition to compartmentalization by conventional organelles, various plant-specific organelles have also been implicated in specialized metabolite synthesis and storage. For example, microscopic examination of tannin-rich tissues from a number of vascular plants identified the tannosome as a new organelle involved in tannin polymerization and trafficking[64]. In these tissues, proanthocyanidin monomers enter chloroplasts and polymerize inside of thylakoids. Tannosomes form by pearling of the thylakoids, bud from chloroplasts, and traffic through cytoplasm to the vacuole where the enclosed condensed tannin is terminally deposited (**Figure 4b**). Likely through a

similar mechanism, dedifferentiated chloroplasts in cells of vanilla fruit give rise to another specialized organelle, the phenyloplast, which accumulates high concentration of 4-*O*-(3-methoxybenzaldehyde) β-d-glucoside, a major phenol glucoside produced by vanilla fruit[65]. Interestingly, vanilla β-d-glucosidase, the enzyme responsible for hydrolyzing 4-*O*-(3-methoxybenzaldehyde) β-d-glucoside to release the sweet-scented aglycone vanillin, was found to localize around phenyloplasts, suggesting a role for phenylplasts in volatile emission in vanilla fruit[65]. In tapetum cells, which are specialized nutritive cells within floral anthers, two types of morphologically distinct organelles—elaioplasts and tapetosomes—have been well observed through classic microscopy studies[66]. Although their particular molecular compositions and biochemical functions remain unknown, it is hypothesized that they evolved to support the biosynthesis of sporopollenin, the hydrophobic inert plant polymer that coats the outer wall of plant pollen grains[67].

Some plant specialized metabolic pathways divide the labor between different cell types. For example, enzymic steps involved in morphine biosynthesis were shown to be distributed between phloem sieve elements, companion cells and laticifers[68] (**Figure 4c**). The natural insecticidal compounds, pyrethrins, which are produced in *Pyrethrum* plants and related *Tanacetum* species, present another example where the biosynthesis involves a multi-organellar and multicellular process. Plastids, endoplasmic reticulum, cytosol, and peroxisomes in ovary trichomes are all involved in the biosynthesis of terpene-derived chrysanthemic acid, which is then transported to the pericarp for methylation and final esterification with a jasmonic-acid-derived alcohol[69].

To achieve their biological functions, many specialized metabolites have to be secreted from the producing cells either to the environment or into specialized storage structures such as trichomes, laticifers, and resin ducts. Active transport of metabolites often relies on members of the ATP-binding cassette (ABC) transporter family. This includes plasma-membrane-localized pleiotropic drug resistance transporters, PDRs, which have been implicated in transport of terpenoids to trichomes in *Artemisia annua*[70], as well as to site of pathogen invasion in *N. benthamiana*[71]. PDR-type transporters can export not only terpenoids, but also other classes of specialized metabolites, such as phenylpropanoid-derived *O*-methylated coumarins that are secreted from *N. tabacum*

roots to the rhizosphere in response to iron deficiency[72]. Additionally, efficient transport of phenylpropanoid/benzenoid volatiles across the plasma membrane in petunia flowers was shown to require an ABCG transporter, the action of which was essential to prevent internal accumulation and cellular self-intoxication[73]. Excretion of specialized metabolites often involves crossing the cuticle, which itself passively sustains the export process by serving as a sink for hydrophobic metabolites[74]. A deeper understanding of the molecular mechanisms and structure-function relationships of metabolite transport and retention, especially in non-model plants, will greatly improve our abilities to engineer their biosynthesis in heterologous hosts.

The elaborate series of enzymatic reactions involved in many plant specialized metabolic pathways requires higher-order organization of the participating enzymes. Instead of relying on diffusion to find their substrates, enzymes of some metabolic pathways have been implicated to form physical assemblies *in vivo*, also known as metabolons, that channel reactive or hydrophobic intermediates to prevent them from being consumed by competing reactions or sequestered by lipid membranes[75]. Early isotope dilution assays conducted on *N. tabacum* cells, together with microsomal assays and colocalization experiments, suggest that PAL and cinnamate 4-hydroxylase (C4H), the first two committed enzymes in general phenylpropanoid metabolism, form a metabolon[76]. By using styrene maleic acid copolymers, the ER-tethered metabolon responsible for the biosynthesis of dhurrin, a cyanogenic glucoside present in *Sorghum bicolor*, was recently isolated, which contains at least four enzymes involved in dhurrin biosynthesis: P450 oxidoreductase (POR), two cytochromes P450 (CYP79A1 and CYP71E1), and and a glucosyltransferase (UGT85B1)[77] (**Figure 4d**). In Arabidopsis, the tryptophan-derived defense compound camalexin is also thought to be produced by an ER-anchored metabolon[78]. As a result, intermediates along the pathway, such as indole-3-acetaldoxime, do not accumulate during camalexin production[78]. The fact that many plant specialized metabolic metabolons are membrane-associated raises the possibility that the membrane itself may also play a role in channeling hydrophobic intermediates[76], although dissecting this possibility from channeling by adjacent enzyme active sites is technically difficult. Recent studies suggested that some metabolons require non-enzyme scaffolding proteins to assemble and function properly. For

example, a pair of membrane steroid-binding proteins (MSBPs) were found to serve as a scaffold to physically organize three monolignol biosynthetic CYPs on the ER membrane in Arabidopsis, likely mediating the formation a lignin biosynthetic metabolon[79]. Characterization of plant metabolons remains challenging, where it is critical to establish i) transient physical interaction between enzymes in a pathway and ii) substrate channeling *in vivo* to identify a metabolon, distinguishing these from other enzyme-enzyme assemblies[80]. Recent advancement in superresolution imaging[81] and proximity labelling techniques, such as the biotin ligase TurboID[82], may help future research of metabolons involved in plant specialized metabolism.

Multifaceted evolutionary mechanisms contributing to the expansion of chemodiversity in plants

The remarkable per-species and collective chemodiversity observed in the plant kingdom suggests that plants must be permissive to evolving new metabolic enzymes. Compared to conserved primary metabolic pathways (e.g. glycolysis and TCA cycle), which have been under stringent selection for billions of years in all life forms, disparate specialized metabolic traits have arisen upon varying selection pressures at different times during the past 470 million years of land plant evolution, resulting in a great number of less perfected extant enzymes. Indeed, large-scale analyses of available kinetic parameters for known enzymes revealed that enzymes of specialized metabolism are on average 30-fold slower than those involved in central metabolism[83]. Perhaps, this general dichotomy between primary and specialized metabolic enzymes is a result of organismal-level selection, which ensures that those less critical specialized metabolic processes do not siphon significant amounts of flux away from more important primary and other secondary biosynthetic pathways.

Early iterations of novel specialized metabolic pathways can arise from enzyme promiscuity intrinsic to the ancestral metabolic system[84]. Promiscuous activities of enzymes may occur at the level of (i) substrates, when an enzyme can perform the same type of reaction on multiple substrates, as is well documented for CYPs, acyltransferases, methyltransferases, glycosyltransferases, and more; (ii) products, when an enzyme could produce different products from the same set of substrates,

commonly observed in terpene synthases, type III polyketide synthases, and other regio- or catalytic-cycle-permissive enzymes; or (iii) catalysis, when one enzyme can catalyze different reaction types, usually in a substrate-dependent manner, exemplified by soybean 2-hydroxyisoflavanone dehydratase which has both dehydratase activity for 2-hydroxyisoflavanone, and esterase activity against ester substrates[85]. Niche-specific evolutionary pressures select for certain promiscuous activities of ancestral enzymes to produce divergent beneficial compounds, charting unique trajectories towards taxonomically restricted specialized metabolism. Motif-enrichment analysis and molecular dynamics simulations of BAHD acyltransferase family members, for example, revealed that such specialization may result in concentrated sequence variations on specific motifs, rather than globally distributed mutations throughout the enzyme structure[86]. Nevertheless, the pressure to evolve new functions often conflicts with the need to preserve the original function of the progenitor enzyme, which is ultimately resolved through gene duplication followed by subfunctionalization/ neofunctionalization[87].

Gene duplication events are caused by genetic aberrations such as replication slippage, retrotransposition, ectopic recombination, aneuploidy, or polyploidy[88], which seem to be better tolerated by plants than other eukaryotic organisms[89]. These serendipitous events sow seeds for metabolic evolution[87]. In recent years, the rapidly growing number of sequenced plant genomes has helped to unveil detailed processes of metabolic innovations following gene duplication events. For instance, copy number variations of metabolic genes were found to be a major contributor to divergence of specialized metabolic traits between the closely related species *Arabidopsis thaliana* and *A. lyrata*[90]. Such increase in copy number of enzyme-encoding genes enables broadened catalytic specificity, thereby potentiating subsequent subfunctionalization or neofunctionalization to occur[91]. In the medicinal plant Chinese skullcap (*Scutellaria basicalensis*), neofunctionalized gene duplicates from the ancestral scutellarein pathway, and at least one subfunctionalized gene, were found to contribute to the novel flavone pathway leading to baicalein and wogonin[92]. Likewise, nepetalactone, an insect-repelling volatile iridoid uniquely found in catnip (*Nepeta spp.*), evolved from a gene duplication event involving an progenitor enzyme with moonlighting iridoid

synthase (ISY) activity, which later gave rise to a dedicated iridoid biosynthetic enzyme[5] (**Figure 5a**). Further formation of the nepetalactone isomers requires the activities of nepetalactol-related short-chain reductase/dehydrogenases. Again, these new enzymes arose via a series of gene duplication events from a single common ancestor followed by subsequent functional diversification, and have co-evolved with the iridoid synthase gene[5]. Recently, the conserved chalcone isomerase in flavonoid biosynthesis was found to be neofunctionalized from a more ancient fatty-acid-binding protein[93], thereby providing a unique example of an enantioselective cyclase that emerged *de novo* from a noncatalytic progenitor.

En route to assemble a new and efficient specialized metabolic pathway, participating enzymes need not only to have the appropriate catalytic functions, but they also have to acquire proper expression patterns that fit the functional needs of the newly evolved pathway. Altering gene expression profiles often involves genetic changes to the promoters and other DNA-regulatory elements that activate and ultimately control transcription of the newly evolved genes in a temporal, spatial, and/or stimuli-dependent manner. New transcriptional regulatory elements may arise by repurposing ancestral elements through single-nucleotide mutation, deletion, insertion or rearrangement, or by de novo evolution. Although these mechanisms have yet to be studied in depth in the context of plant metabolic evolution, the former was recently highlighted in *Drosophila melanogaster*[94]. It appears that mutations in both cis and trans elements could occur over relatively short evolutionary timescales and contribute to differential expression profiles of orthologous genes in different genotypes[94]. To explore the potential of de novo evolution, the lac operon promoter in *E. coli* was replaced with random 103-bp-long sequences, which revealed that approximately 10% of these sequences are capable of eliciting transcription, and an additional 60% are only one mutation away from being active promoters[95]. It is not known yet whether such level of permissiveness to alter existing promoters or generate novel ones prevails in plants, but it would provide the necessary evolutionary flexibility for diverse plant specialized metabolic pathways to gain optimal expression patterns specifically suited for their functions.

It is well established that prokaryotes and fungi contain operons or gene clusters in their genomes to facilitate co-regulation of genes participating in the same biological

process. In plants, as in other eukaryotes, the absence of functional operons and the prevalence of mechanisms that act to disperse genes (translocation, inversion, and unequal crossing over) gave little reason to expect clustering of co-functioning genes. While this expectation still holds true in general, the rapidly expanding plant genomic resources have revealed that co-functioning specialized metabolic genes can form gene clusters in plant genomes[96]. These observations have also led to the development of bioinformatic tools that predict unknown metabolic pathways in a sequenced genome based on gene clustering patterns[97]. One explanation for the existence of plant specialized metabolic gene clusters is the necessity of co-segregation of these genes in a population to maintain the overall integrity of the pathway. However, recent studies also uncovered the role of gene clustering in coordinated transcriptional regulation. Known gene clusters across multiple plant species exhibit distinct chromatin signatures, which implies that genes on the same gene cluster—sometimes over long chromosomal distances—could be brought together through specific three-dimensional chromosomal topologies to facilitate co-regulation[98] (**Figure 5b**). Through this mechanism, the host plant could more effectively suppress multiple genes of a specialized metabolic pathway when in resting state or in an inappropriate tissue, and co-activate their expression when condition warrants[99].

Future perspectives

The rapidly expanding tool sets in chemistry, genomics, molecular and synthetic biology now have facilitated exploration of new territories in plant specialized metabolism at an unprecedented pace. These efforts have already started to expand into the rich biodiversity of non-model plants, including those harboring interesting chemistry and bioactivities, and crop plants cultivated and utilized by populations around the world. Beyond elucidating the genetic and biochemical makeup of specialized metabolic pathways, higher-level adaptive mechanisms involved in these metabolic processes as discussed in this review will continually be uncovered, thus contributing to a comprehensive understanding of metabolism as an integral part of organismal biology. Moreover, comparative studies of plant specialized metabolism especially in closely related species will provide fertile ground for researchers to probe how enzymes acquire

new activities and subcellular localizations, how multi-step metabolic pathways are assembled, and how gene regulatory networks controlling these pathways emerge. Ultimately, the field will establish mechanistic links between the multitude of plant metabolic traits and the biological functions they play in host plants under dynamic biotic and abiotic conditions. Such knowledge will be critical for translational applications of plant specialized metabolism in various arenas such as drug discovery, agriculture and biotechnology. On the technological front, a quantitative multi-omics approach with enhanced throughput and resolution (e.g., at tissue and cellular levels), the ability to genetically manipulate non-model plants at will, and plant cell/tissue culture biotechnologies are among the few frontiers that will likely yield transformative opportunities for plant specialized metabolism research in the coming years.

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J.K.W. is a member of the Scientific Advisory Board and a shareholder of DoubleRainbow Biosciences, Galixir and Inari Agriculture, which develop biotechnologies related to natural products, drug discovery and agriculture. All other authors have no competing interests.

Figures

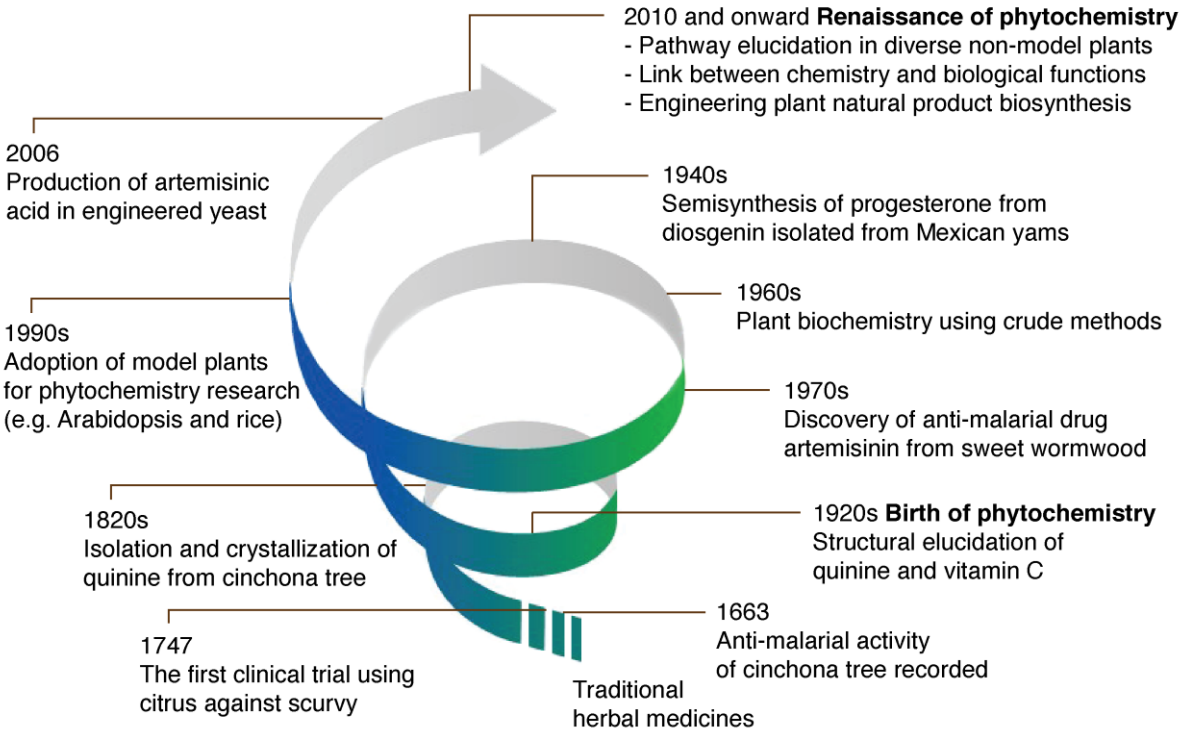

Fig. 1 | A brief history of phytochemistry research. Select milestones in humans' exploration of plant chemistry and biochemistry using modern scientific methods since the 17th century are denoted on a spiral timeline. Prior to modern science, humans have harnessed the medicinal properties of plants for millennia. The field started as a subdiscipline of chemistry, and progressed into biochemistry, molecular genetics and bioengineering in the following decades, often propelled by technological advances in these fields. The selected advances are representative of a greater number of achievements that were made during each era.

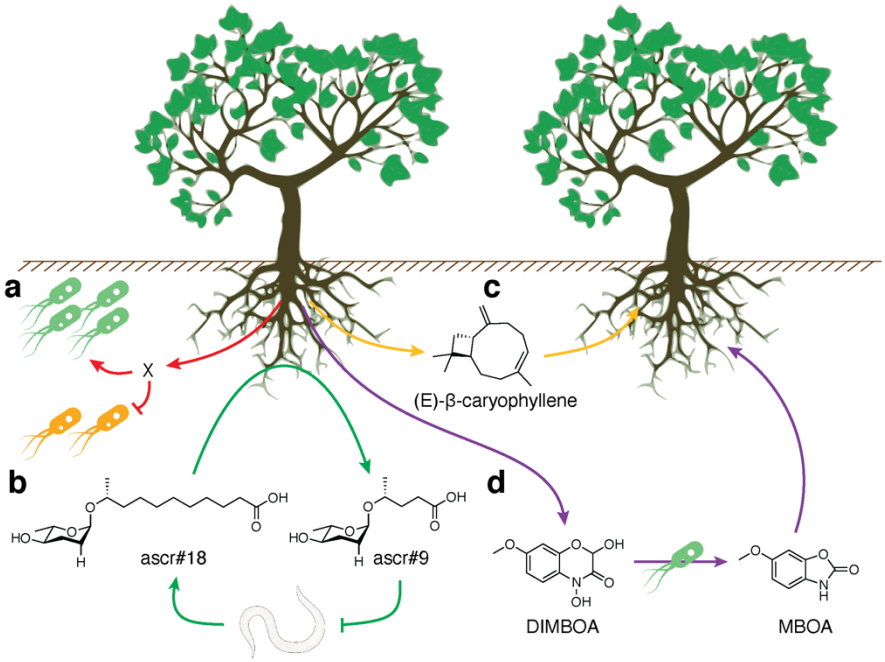

Fig. 2 | Modes of below-ground biotic interactions mediated by plant specialized metabolism. a, Influence of root-secreted metabolites (e.g. flavonoids) on promoting colonization of beneficial microbes while inhibiting colonization of detrimental microbes. **b**, Plant metabolic enzymes use non-plant substrates in the synthesis of repellants of attacking species[15]. **c**, Secreted metabolites act as signals to neighboring plants, either directly, or **d**, after modification by the rhizosphere microbes[16]. DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; MBOA, 6-methoxy-benzoxazolin-2-one.

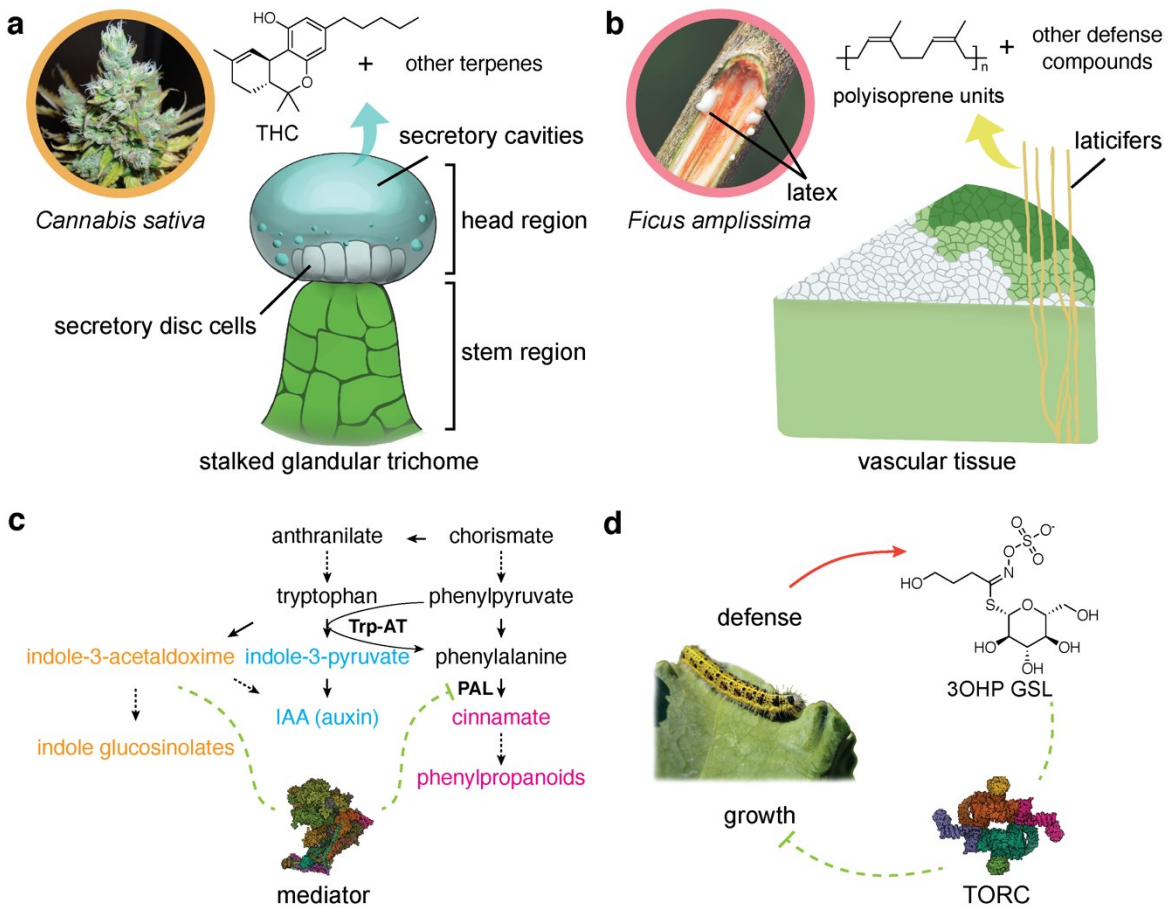

Fig. 3 | Integration of plant specialized metabolism with body plan adaptation and growth regulation. a, The stalked glandular trichome is a highly adaptive tissue in *Cannabis sativa* that biosynthesizes cannabinoids and other terpenes and stores them in the secretory cavities in the head region. THC, tetrahydrocannabinol. **b**, Laticifers, specialized secretory cells in the vascular tissues of many plants, including *Ficus amplissima*, accumulate and ooze out latex upon injury. Latex contains polyisoprene units and other lineage-specific defense compounds. **c**, An example of interconnection of metabolic and signaling networks, where the indole glucosinolate and phenylpropanoid pathways are linked to production of auxin, regulating plant growth according to the metabolic status of these pathways. IAA, Indole-3-acetic acid; Trp-AT, Tryptophan aminotransferase; PAL, Phenylalanine ammonia-lyase. **d**, Certain glucosinolates produced in response to insect herbivory in Brassicales can regulate plant growth through the TOR signaling pathway. 3OHP GSL, 3-Hydroxyl-propyl glucosinolate; TORC, Target of rapamycin complex.

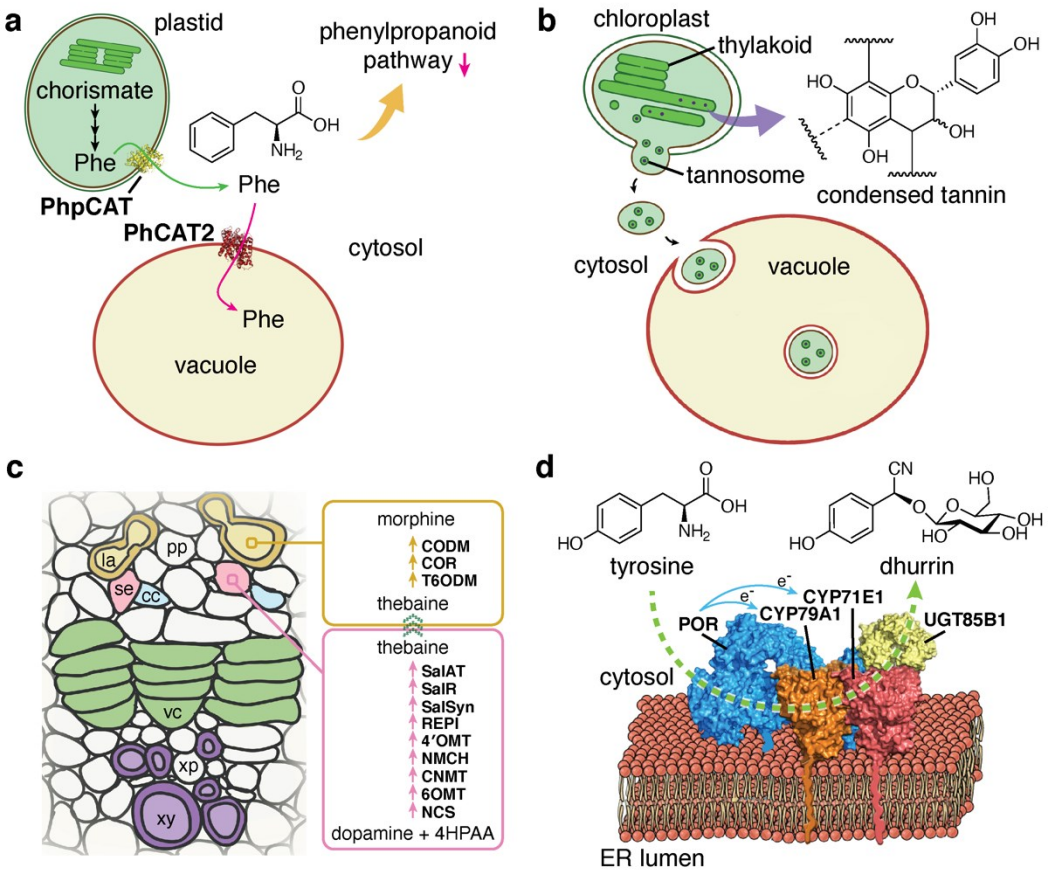

Fig. 4 | Contribution of subcellular and intercellular mechanisms to optimal multifunctionality of plant specialized metabolic processes. a, Phenylalanine (Phe), the precursor of plant phenylpropanoid metabolism, is biosynthesized in plastids and transported through PhpCAT to cytosol[58]. Excess Phe can be sequestered into vacuole by PhCAT2[59]. **b**, Formation of tannosomes from redifferentiated chloroplasts. Tannosomes containing condensed tannins and other polyphenols are trafficked to and stored in vacuole. **c**, Cross section of the vascular tissue of opium poppy illustrating the partitioning of morphine biosynthetic pathway enzymes into laticifers (la) and the neighboring sieve elements (se). The companion cells (cc) also contribute to production of morphine biosynthetic enzymes, which are transported into laticifers and sieve elements[68]. pp, parenchyma; vc, vascular cambium; xp, xylem parenchyma; xy, and xylem vessels. **d**, Assembly of the dhurrin metabolon at the cytosolic face of ER membrane in sorghum[77].

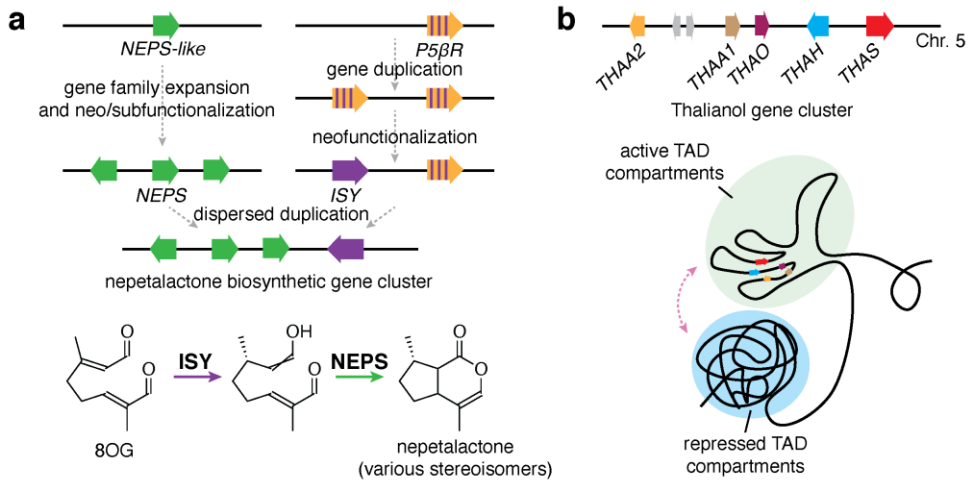

Fig. 5 | Evolutionary mechanisms contributing to novel plant specialized metabolic traits. a, The evolutionary trajectory underlying the occurrence of nepetalactone biosynthesis in catnip involves multiple gene duplication events followed by neo/subfunction of a promiscuous *P5βR* ancestral gene and a NEPS-like gene[5]. The promiscuous ISY activity in P5βR is depicted as purple stripes. Several NEPSs contribute to the production of various stereoisomers of nepetalactones. ISY, Iridoid synthase; NEPS, Nepetalactol-related short-chain reductase/dehydrogenases; P5βR, Progesterone 5β-reductase; 8OG, 8-Oxogeranial. **b**, Co-regulation of the biosynthetic genes of the thalianol gene cluster in Arabidopsis is facilitated by specific three-dimensional topologies of their encompassing chromosome[98,100]. These genes are likely connected via topologically associating domain (TAD) compartments of chromosome 5 to coordinate their expression in root cells. In contrast, association of the thalianol gene cluster with repressed TAD compartments is correlated with suppressed expression, for example in leaves. THAA, thalianol acyltransferase; THAO, thalianol oxidase; THAH, thalianol hydroxylase; THAS, thalianol synthase.

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Leveraging coexpression analysis and transient transformation of *Nicotiana benthamiana*, the authors identify the biosynthetic pathway of the etoposide aglycone podophyllotoxin in mayapple.

Using a comparative phylogenomics approach, the authors delineate the process underlying the re-evolution of iridoid biosynthesis in catnip in the Nepeta lineage, which involves the assembly of a nepetalactone biosynthetic gene cluster.

This work demonstrates that the tomato rhizosphere microbiome affects the chemical composition of root exudation through a systemic root–root signaling mechanism dubbed systemically induced root exudation of metabolites (SIREM).

Analysis of the impact of indole glucosinolate intermediates on flux towards production of phenylpropanoids reveals the intertwined roles of metabolism, transcriptional control, and protein turnover on co-regulation of distinct metabolic pathways.

Glucosinolates are specialized defense compounds produced by Brassicaceae plants against herbivores. This work identifies one of these glucosinolates, 3-hydroxypropylglucosinolate, regulates root growth through influencing the TOR complex.

A carotenoid-derived dialdehyde (diapocarotenoid) is identified as the specific signal needed for anchor root formation in Arabidopsis.

This work reveals the hormone-like function of terpenoids and their aerial transport within enclosed spaces of plant tissues, which impacts organ development and reproductive fitness.

This work shows that emission of volatile compounds out of the cells relies on active transport and requires the action of an ATP-dependent transporter.

By employing styrene maleic acid copolymers, this work identifies the metabolon that produces the cyanogenic glucoside dhurrin in *Sorghum bicolor*.

This work reveals that plant biosynthetic gene clusters reside in highly interactive chromosomal domains that undergo marked changes in local conformation and nuclear positioning as a mechanism for coregulation of genes on the cluster.