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A plant balancing act: Meshing new and existing metabolic pathways towards an optimized system

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Abstract

Specialized metabolic pathways evolve from existing pathways, creating new functionality potentially boosting fitness. However, how these pathways are integrated into a pre-existing working and well-balanced metabolic system is unclear. They could be integrated to the system as a functional appendage, or they could be fully embedded into primary metabolism by establishing new biochemical and regulatory connections. A full integration into the primary metabolic system requires substantial system re-wiring and because of this complexity, the latter is often not experimentally pursued. New studies provide evidence that some specialized metabolic pathways are fully embedded in primary metabolism with extensive new regulatory and biochemical connections. This suggests, that we should consider whether other specialized metabolic pathways could be fully integrated rather than being simple appendages. In this mini review, we survey compelling evidence supporting that some specialized metabolic pathways are fully integrated and ask if these metabolites now act as de-facto primary metabolites?

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Retrograde metabolic flow, Primary metabolism, Specialized metabolism, Dual role enzymes, Glucosinolate, Integrated/coordinated metabolism, Broad TF regulation.

Abbreviations

Gls, Glucosinolates; S-TF, specialized transcription factors.

Introduction

We often parse plant metabolism into primary and secondary/specialized metabolism. Classically, this division is guided by primary metabolism being considered essential for intrinsic processes like energy conversion, replication, transcription, etc. that are necessary for survival across all environments. In contrast, secondary/specialized metabolism may be essential in only specific environments and provides the ability to adapt to varying environments. This reasoning can lead to the idea that specialized metabolism is generally non-essential, but this is only true if the specific environment to which a specialized metabolite provides adaptation is never encountered or studied. Another outgrowth of this thinking is that primary metabolism is typically assumed to be older and more conserved leading to the assumption that primary metabolism shows more interconnections, to maintain system and metabolic stability [1]. In contrast the younger specialized metabolism pathways arise through whole-genome or tandem duplications and ensuing neo-functionalization with the recruitment of existing enzymes or pathways, from existing primary or specialized metabolism [2–4]. Recently, the specialized and generalized terms are reoriented more toward their age and frequency of occurrence [5,6]. In combination, the summation of the above ideas leads to a model whereby specialized metabolism regulation and interconnection to the rest of the organism is often considered looser and as such, potentially easier to manipulate by engineering or moving to a naïve species [7–9]. However, parts of primary metabolism can evolve on short time-scales like specialized metabolism as indicated by the rapid evolution of C2/C3/C4 photosynthesis within and between plant species across the Viridiplantae [10,11]. Further, recent work is beginning to show that specialized metabolites may have tight biochemical and regulatory connections similar to that observed for primary metabolism. The assumptions and observations around this pathway/metabolite evolution generate two potential scenarios of how novel metabolites can integrate into the metabolic system (see Box 1): in Scenario 1, the metabolite is added to the metabolic network as a peripheral node, while in scenario 2, the metabolite is fully integrated into the system, potentially by considerable system re-wiring. Flux connections that allow novel specialized metabolites to be recycled back into primary metabolism and considerable system re-wring would be

necessary for Scenario 2. Regulatory connections, including deep regulatory co-ordination and potentially enzyme sharing, would also be necessary. In this mini review, we focus on evidence supporting the existence of the second scenario. We describe recent studies identifying several distinct forms of flux connections including direct retrograde flux of specialized metabolites to create primary metabolites, shared enzymes, and regulatory co-ordination. These exciting findings highlight the need to more broadly assess when and which pathways exist under these different scenarios.

Box-1: The theoretical framework: how can a novel metabolite be integrated into a cellular and metabolic network?

Scenario 1: *A novel functional metabolite is added to a metabolic and cellular network as a peripheral appendage.* In this scenario, limited biochemical and regulatory connections to primary metabolism are established, allowing the new metabolite to be quickly regulated by the evolution of one or a few transcription factors (Figure 1). Further, the low connection to primary metabolism means evolutionary loss or change in this metabolic pathway will minimally disrupt the rest of the plant's physiology or metabolism. An advantage for the plant is that this scenario may enable a fast diversification of the novel metabolic pathway by minimizing constraint. Limited metabolic integration into the system does present a potential disadvantage by a unidirectional flow of information, energy and elements from primary to specialized metabolism, which could be wasteful.

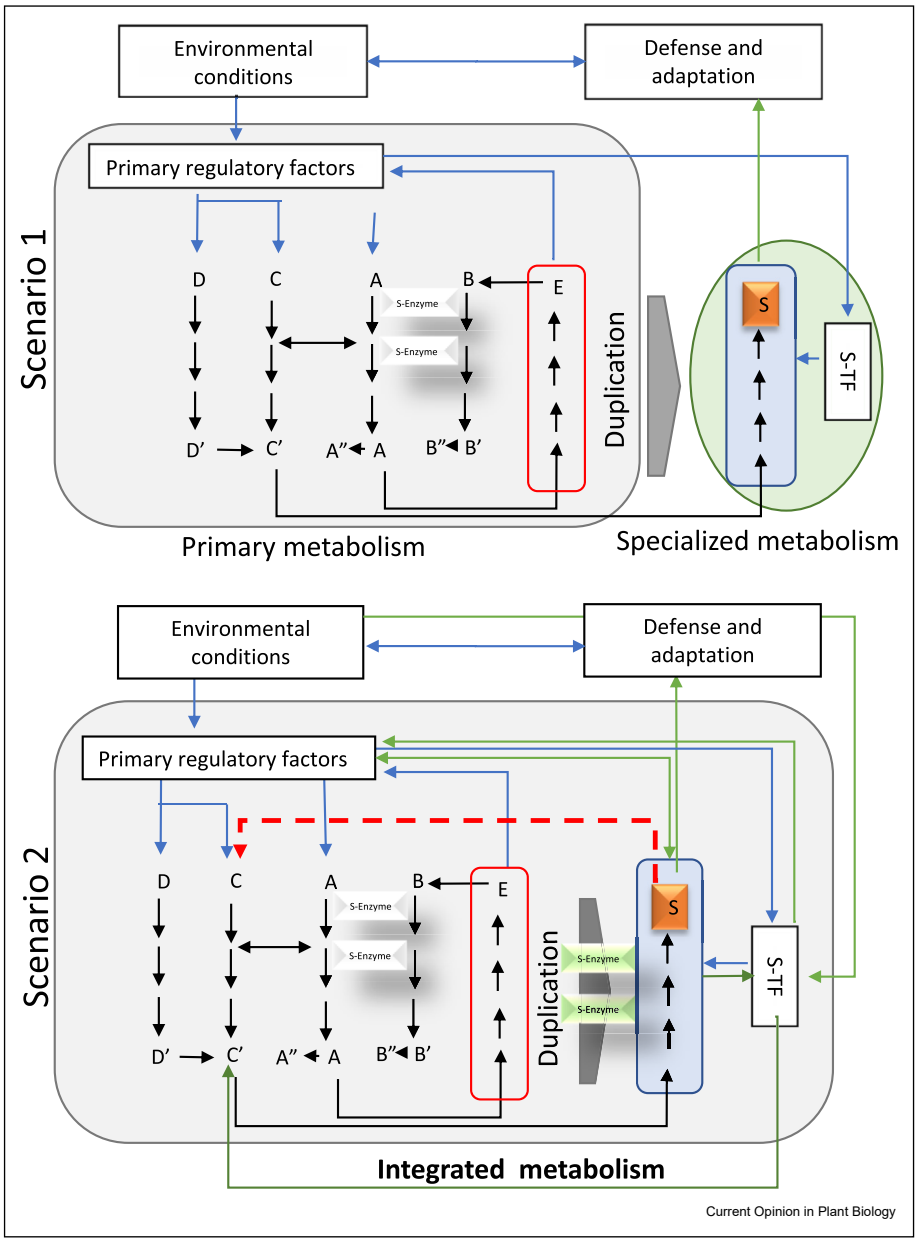
Scenario 2: A novel metabolite is fully integrated into the system via extensive biochemical and regulatory connections to become an integral part of metabolism. Under this scenario, metabolic resources can be reallocated back to primary metabolism and an integrated regulatory network is established to balance the operation of the system (Figure 1). Such a scenario is potentially efficient from an energetic and operational perspective, but it requires an understanding of how a fully-functioning system can evolve to allow the establishment of these new connections. The involvement of specialized metabolism in growth, development and signaling can be rationalized under such a scenario, but it also implies that removal or evolutionary change of the fully integrated metabolite is difficult due to the integration.

When or how long each scenario takes place is unclear and could be a function of time since the novel metabolite arose. Other factors could also influence when pathways occupy one or the other pathway. For example, the integration of metabolites that are sequestered to a specific tissue or inactive structure because they are bioactive or toxic may be different than those that are not sequestered [12–15]. One possibility is Scenario 1 represents a transient step with ensuing selective processes potentially creating new interconnections that lead to Scenario 2 if fitness is boosted.

Retrograde flow from secondary to primary metabolism

Retrograde flow from specialized metabolism back to primary metabolism has been long hypothesized and supported by indirect evidence. This retrograde flow is represented by the red arrow in Figure 1 Scenario 2. For example, many specialized compounds decrease in response to nutrient stress [12], implying a reallocation back to primary metabolism. Direct enzymatic or molecular evidence for such biochemical reallocation is just becoming available. A recent study by Ref. [16] provided experimental evidence and the potential mechanism by which one sulfur in glucosinolates (GLs) can "retrograde" flow to the free amino acid biosynthesis such as cysteine (Cys) in Arabidopsis. GLs are sulfur and nitrogen specialized metabolites that are largely limited to the order Capparales and provide a number of adaptive benefits to the plant under a myriad of biotic and abiotic environments [17–19]. The original GL pathway evolved using the CYP450s from cyanogenic glucosides in combination with genes from other primary and specialized metabolite pathways. Additional whole genome duplications within the Capparales allowed for neofunctionalization of the whole pathway to create new classes of GLs [4]. Several studies suggest that GL catabolism can provide sulfur to primary metabolism during sulfur starvation. Consistently, a large fraction of sulfur assimilation in Arabidopsis is co-regulated with GL production [20,21] and GL synthesis is down-regulated under low-sulfur conditions [22]. The retrograde flow of sulfur to primary metabolism is also consistent with the hypothesis that GLs partly function as a sulfur storage. This hypothesis is supported by the induction of the GLs breakdown upon broccoli (*Brassica oleracea* var italic) and Arabidopsis seed germination, where GLs are a major form of sulfur storage ([23,24]. A knockout of the two GL transporters (GTR1 and 2) responsible for GL transport to seeds [25] leads to the elimination of seed GLs and a 90% decrease in seed sulfur content [26]. Furthermore, elimination of only the aliphatic GLs, the most prevalent type in Arabidopsis seeds [23], using a double knockout mutant of the two specific TFs of this pathway, MYB28 and MYB29 [27], creates a similar sulfur reduction in seeds [26]. Thus, in addition to providing seedling biotic defense, GLs may be a critical sulfur source for the developing crucifer seedling during starvation.

Figure 1

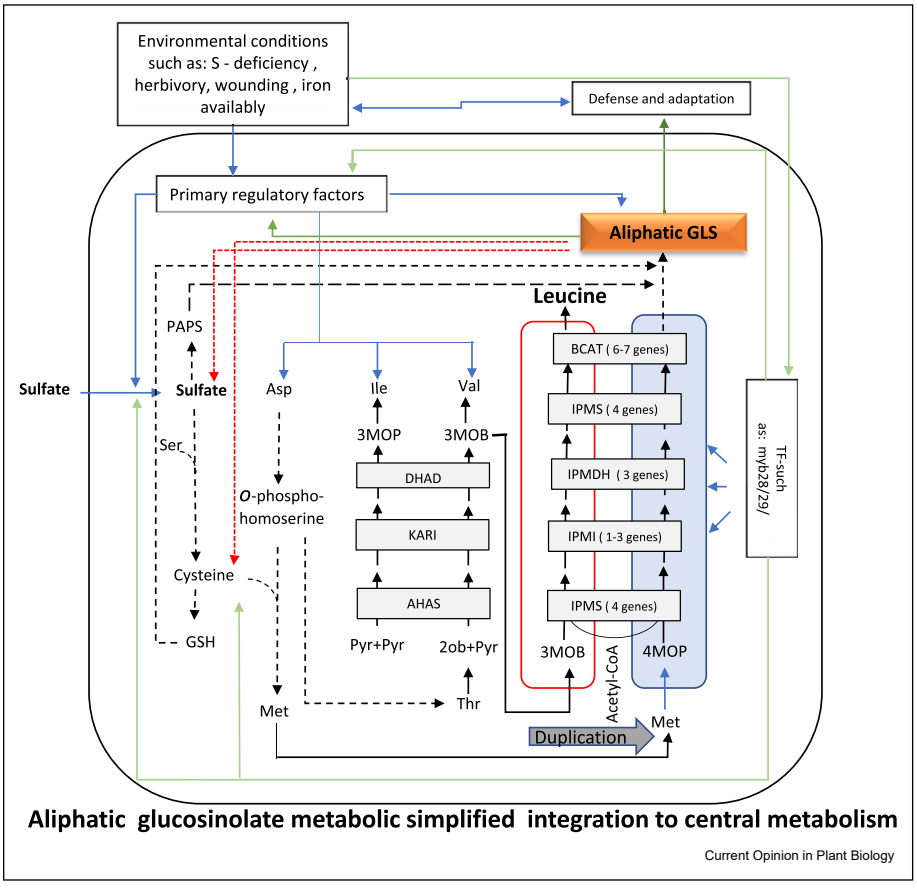


legend: Schematic comparison of Scenarios 1 and 2. Illustration of hypothetical primary metabolic pathways A–E. A pathway leading to the production of S (specialized metabolite) may result from the duplication of the primary metabolic pathway E following a whole genome duplication event. The original primary pathway is marked with a red box and the duplicated specialized metabolic pathway is marked with a blue box. Under Scenario 1, the duplicated pathway is added as an appendage akin to a "stand alone" metabolic module (marked as a green oval). Metabolic resources flow into the pathway and regulation is hierarchical. The output and function of S under such a scenario is largely related to defense or fitness under a specific environment. Black arrows represent metabolic flow. Blue arrows represent informational/signaling flow. Green arrow represents novel connections resulting from the newly added metabolic pathway. S-TF, specialized metabolic pathway transcription factor. Under Scenario 2, the duplicated pathway may share enzymes (S-enzyme) with the parent pathway. The end product of the novel pathway or its intermediates can be re-cycled back to the primary metabolism represented by the red dotted arrow. The metabolites emerging from this pathway may function to enhance fitness and adaptation but can be additionally integrated to promote growth and control development. Novel connections resulting from the newly added metabolic pathway and establishment of new regulatory systems under Scenario 2 are presented by the green arrows. Under Scenario 2, TFs related to both primary and/or specialized metabolism may respond to shifts in primary metabolism, environmental conditions and the intermediates or end products of the pathway leading to S. TFs also have a broader impact on both types of metabolism in Scenario 2.

Only recently has at least one mechanism been identified to allow for this recycling [16]. used labeled GL tracer experiments to demonstrate that at least two of the three sulfur atoms from the aliphatic GL 4-methylsulfinylbutyl are mobilized toward primary metabolism under sulfur deficient conditions: one atom can be released through SO_4^{2-} and another ends up in primary metabolism for Cys biosynthesis. The metabolic pathway leading to the reintegration of the GLs' sulfur to Cys requires the activity of two beta-glucosidases (BGLU28 and BGLU30) that are specific to the youngest class of GLs, the aliphatic GLs. BGLU28/30 enzymes were not tested to assess if they evolved before or after the aliphatic GLs within the Capparales. Because the sulfur atom tested for recycling is unique to the aliphatic GLs, testing the age of the BGLU28/30 enzymes would provide insight into how quickly the ability to recycle this sulfur evolved following the evolution of the new aliphatic GLs. A simplified illustration of the aliphatic GL metabolic pathway integration into central metabolism is in Figure 2, where the red dotted arrows represent sulfur recycling.

Another example of the recycling of a specialized metabolite family back to primary metabolism is seen in cyanogenic glycosides. This is a group of nitrile-containing specialized metabolic compounds yielding cyanide upon glucose cleavage. They are wide yet sporadically distributed in higher plants and are predominantly known as an anti-herbivory plant defense [28]. Some studies hint at an additional role as a key transportable form of reduced nitrogen [29–31]. In

Figure 2



A schematic illustration of key aspects of the aliphatic GLs metabolic integration in the context of the Scenario 2 framework. Key primary metabolic pathways are illustrated. Leucine biosynthesis is marked with a red box. The Leu metabolic biosynthetic pathway, which was duplicated and evolved to become the aliphatic GL biosynthetic pathway, is marked with a blue box. Potential enzyme sharing between Leucine and aliphatic GLS is illustrated in the shaded boxes. Black arrows represent metabolic flow. Dotted black arrows represent metabolic flow requiring multiple enzymatic steps. Blue arrows represent informational/signaling flow. Green arrows represent newly formed regulatory connections resulting from the newly added metabolic pathway. The red arrows represent the retrograde metabolic flow from aliphatic GLs back to primary metabolism. Due to the high complexity of the integrated metabolome, only some metabolic pathways, regulatory connections and TFs involved are included, and only some representative aspects are illustrated. PAPS: 3′-phosphoadenosine 5′-phosphosulfate; 3MOP: 3-Methyl-2-oxopentanoate; 3MOB: 3-Methyl-2-oxobutanoate; DHAD: dihydroxyacid dehydratase; KARI:, ketolacid reducto-isomerase; AHAS: acetohydroxyacid synthase; Pyr: pyruvate; Ob: oxobutanotate; BCAT: branched-chain amino acid aminotransferase; IPMS: isopropylmalate synthase; IPMI: isopropylmalate isomerase; IPMDH: 1sopropylmalate dehydrogenase; 4MOP: 4-Methyl-2-oxopentanoate; GSH: glutathione; Asp: aspartate; Ile: isoleucine; Val: valine; Met: methionine; Thr: threonine.

cassava, the cyanogenic glycoside Linamarin can be converted to asparagine to provide a major transportable form of reduced nitrogen from leaves to roots, where it is stored and used for amino acid synthesis [32–35]. Linamarin catabolism leads to the generation of cyanide, which is then assimilated via β-cyanoalanine synthase to produce β-cyanoalanine and sulfide [36]. Following this step, β-cyanoalanine hydration form asparagine, which plays a primary role in nitrogen recycling, storage and transport in plants [37]. The deamination of asparagine leads to aspartate and free ammonia that can be reassimilated by the glutamine synthase cycle and used for the synthesis of multiple amino acids [38].

Additional possible retrograde connections have been suggested by the potential of more than one catabolism pathway to recycle both cyanogenic glycosides and glucosinolates. Interestingly, these additional pathways may share a related chemistry. In cyanogenic glycosides, the intact cyanogenic glucoside dhurrin can have the glucose swapped for a glutathione, which can be removed by a lambda class glutathione -S-transferase (GST) to produce a nitrile, *p*-hydroxyphenyl acetonitrile, without cyanide release [39]. Nitrilases can convert the nitrile to *p*-hydroxyphenylacetic acid and ammonia for direct recycling into primary metabolism. This process appears to be widespread: nitrile, amide and carboxy forms of cyanogenic glucosides can be found in cassava, almond and sorghum [40]. Interestingly, the cyanogenic glucoside pathways in these species likely evolved convergently [6], suggesting that this recycling pathway might also have evolved convergently. Supporting this possibility is that glucosinolates can also be converted to nitriles via a family of nitrile specifier proteins [41–43]. These glucosinolate derived nitriles can then be catabolized by a family of nitrilases, convergent to the cyanogenic nitrilases, to a similar suite of amides and carboxylic acids that are ready to go into primary metabolism [44,45]. Together, the data on cyanogenic glucosides and glucosinolates suggests that specialized metabolites can have more than one retrograde link to primary metabolism.

In addition to the cyanogenic glucosides and glucosinolates, flavonoids and monoterpenes are also known to be recycled into primary metabolism. A recent study shows retrograde flow of carbon from flavonoid compounds to ubiquinone, potentially via heme-dependent oxidation [46]. In this example, the B ring of the flavonoid kaempferol can be directly released to form 4-hydroxybenzoate for ubiquinone production. It is still unclear how or if the rest of the flavonoid is utilized. Another system with evidence for recycling is highly abundant foliar monoterpenes within the Lamiaceae [47]. Upon flowering in both peppermint (*Mentha* × *piperita*) and garden sage (*Salvia officinalis)*, the monoterpenes enthrone and camphor are converted into water-soluble transport forms that are exported to the root [48]. Within the root, these transport forms are oxidatively degraded to produce acetyl-CoA, which can then re-enter primary metabolism [49]. While the chemistry of monoterpene recycling is well established, the genetic basis has yet to be determined.

Enzymatic sharing between specialized and primary metabolism

One process giving rise to new specialized metabolism is the recruitment of primary metabolic enzymes, as happened for pyrrolizidine, tropane, acridone and benzoxazinoid alkaloids, chain-elongated glucosinolates, acylsugars and triterpenoids saponin [50]. This process is not clean, as the recruited enzyme can have the same function in the new pathway and in the primary metabolism pathway. Such dual-role enzymes are essential for the formation of chain-elongated aliphatic GLs that emerged as a result of the evolution of methylthioalkylmalate synthases (MAMs) from isopropylmalate synthase (IPMS), an enzyme in the leucine biosynthetic pathway. MAMs catalyze the first committing step in the side chain elongation of methionine derived aliphatic GLs and have a reduced IPMS activity in leucine biosynthesis [2,51]. MAM/IPMS function within a three enzyme cycle along with isopropylmalate isomerase (IPMI) and isopropylmalate dehydrogenase that function for both primary and specialized metabolism [52]. [52] showed that the IPMI large subunit has a dual role:, it participates in leucine biosynthesis when paired with the IPMI small subunit 1 (SSU1), but participates in methionine chain elongation when paired with IPMI SSU2 or 3 [52]. However, later studies showed that Arabidopsis SSU2 and 3 may have residual activity in leucine synthesis [53], and SSU1 is involved in the first cycle of chain elongation [54]. The intricate sharing of enzymes between the two pathways is also seen in the enzymes directly upstream of the MAMs, the branched chain amino acid transaminases (BCATs). BCAT3 functions in both branch chain amino acid synthesis as well as met-derived GLs [52,55]. The enzyme sharing between the biosynthesis of leucine and the GLs is illustrated in Figure 2. Could the dual function of these enzymes be a feature of the system that results in better system regulation and resource allocation? Such a hypothesis aligns with elevation of GLs upon perturbation of the leucine catabolic enzyme, the isovaleryl-CoA dehydrogenase (IVD). A null mutant of this enzyme causes elevation of 12 free amino acids in seeds as well as an increase in methionine and isovaleroyloxypropylglucosinolate along with a reduced 3-benzoyloxypropylglucosinolate, highlighting the tight crosstalk between these two pathways [56]. This raises the question as to whether this phenomenon is a transient stage that will be eliminated in time or a feature that is maintained to allow the interconnection of primary and specialized metabolism.

Regulatory connections

Another mechanism to integrate specialized metabolites into an existing system are regulatory connections that link the new pathway to core primary metabolism and other cellular processes. These regulatory connections can ensure effective optimization of defense under changing environmental conditions. Here, we focus on transcriptional components of this regulatory integration, since the potential for specialized metabolites to function as regulatory compounds has been detailed in other reviews [57–59]. Transcriptional regulation of specialized metabolite pathways is often thought to involve master regulator transcription factors specific to the pathway of interest. The aliphatic glucosinolate pathway has several TF families that fit this model, i.e., MYB28/29/76 and SDI1/2 [20,22]. These TFs evolved coincidentally with the glucosinolates to regulate not only the glucosinolate biosynthetic pathway but also broad swaths of primary metabolism, including most components of sulfur metabolism. This extends beyond sulfur metabolism, as MYB29 also plays a role in controlling nitrogen metabolism, core circadian clock genes and root development in response to nitrogen [60]. Supporting this ability of specialized metabolite TFs to broadly influence primary metabolism, MYB28 has the ability to interact with core components of central carbon metabolism and to control the expression of seed storage proteins in the developing seed [61,62]. Finally, both MYB28 and 29 modulate the iron starvation response independent of their glucosinolate function [63]. Genomic analysis of primary metabolic pathways in Arabidopsis via Yeast-1-hybrid indicates that this broad pathway effect is not glucosinolate specific but is instead a common feature of transcriptional regulation where most plant TFs appear to coordinate across a broad swath of metabolism, both primary and specialized [62]. As such, transcriptional regulation within the glucosinolate pathway coincidentally arose along with the new specialized metabolism pathway and quickly captured the ability to integrate with primary metabolism. Some aspects of the newly formed regulatory connections of MYB28/29 with central metabolism are illustrated in Figure 2).

Similarly, TFs influencing specialized metabolism in petunia aroma production also coordinate across primary metabolism. The R2R3-MYB, EMISSION of BENZENOIDS II (EOBII), regulates the shikimate pathway by controlling the MYB factor ODORANT1(OD1) and several phenylpropanoid scent-related structural genes [64,65]. In poplar, MYB165 regulates genes in the flavonoid, anthocyanin, PA biosynthesis and shikimate pathways [66]. In both petunia and poplar, the MYBs are coordinating a specialized metabolism pathway with the primary metabolism pathways needed to make the requisite precursors. It remains to be seen how often TFs that are considered specific to a specialized metabolic pathway also integrate with the metabolically necessary primary metabolism pathways.

Conclusions

The aforementioned evidence leads us to propose that, as they evolve, specialized metabolic pathways may shift from an initial appendage state (Scenario 1) to become highly integrated into an organism's system (Scenario 2). This integration can involve retrograde flow back to primary metabolism, enzyme sharing and regulatory feedback loops. If most pathways are highly integrated, then this would suggest that engineering a specialized metabolite pathway from one species into a naïve plant host will require significant systems engineering to adjust these connections. Further, as the level of integration increases, it will create increasing contingencies that could prevent a specialized metabolic pathway from being discarded due to associated ramifications to the whole system. For example, only two species of Koberlinia in the entirety of the Cappareles are known to have lost the glucosinolate pathway. Testing these ideas will require a broader survey of specialized metabolic pathways to assess if they fit scenario 1 and/or 2 better. Conducting this analysis across a range of specialized metabolic pathways would allow an assessment of factors that may influence integration, such as pathway age, compound accumulation, ecological role, storage location or other factors that could ultimately affect this process.

Box 2: How does Scenario 2 possibly alter research beyond specialized metabolism?

Population genetics models suggest that pleiotropy (one gene influencing multiple traits) should be selected against due to potential antagonistic effects on fitness. In contrast, Scenario 2 predicts that pleiotropy could be a core component of natural variation as specialized metabolite pathways. Supporting this prediction is a recent GWAS of free amino acids in Arabidopsis seeds that shows that the causal loci for aliphatic GLs also influences free amino acid homeostasis, most prominently free Gln levels [26]. Similarly, including single nucleotide variation in the genes for specific specialized metabolite pathways significantly improves genome prediction for free amino acids in Arabidopsis seeds [67]. Finally, causal loci in aliphatic GLS also influence drought resistance and circadian clock oscillations in Arabidopsis and Boechera [68]. It remains to be seen how many other specialized metabolite pathways show similar levels of pleiotropy and how potential antagonistic effects on fitness affect the plant.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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