ELSEVIER

Metabolon formation and metabolic channeling in the biosynthesis of plant natural products

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Metabolon formation and metabolic channeling in plant secondary metabolism enable plants to effectively synthesize specific natural products and to avoid metabolic interference. Channeling can involve different cell types, take advantage of compartmentalization within the same cell or proceed directly within a metabolon. New experimental approaches document the importance of channeling in the synthesis of isoprenoids, alkaloids, phenylpropanoids, flavonoids and cyanogenic glucosides. Metabolon formation and metabolic channeling in natural-product synthesis facilitate attempts to genetically engineer new pathways into plants to improve their content of valuable natural products. They also offer the opportunity to introduce new traits by genetic engineering to produce plant cultivars that adhere to the principle of substantial equivalence.

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Introduction: why metabolons and metabolic channeling?

To optimize growth and development, the metabolic activities of a plant are highly coordinated at the whole-plant, organ, tissue, cellular, organellar and molecular levels. At the cellular level, channeling of substrates to their target enzymes is facilitated by the compartmentation of the cell into different organelles and sub-structures thereof. This serves to co-localize and optimize the concentrations of enzymes and their substrates. At the molecular level, yet another increase in substrate concentration is gained by the formation of metabolons (i.e. multienzyme complexes). There may be several reasons for metabolon formation. First, to improve catalytic efficiency by channeling an intermediate that is formed at one active site of an enzyme to the active site of the next enzyme, i.e. to bring co-operating active sites into close proximity and thereby decrease the transit time for intermediates. Second, to relieve kinetic constraints that result from the dilution of intermediates into the bulk phase of the cell. Third, to secure swift conversion of labile and/or toxic intermediates into more stable and less toxic constituents by sequestration and by preventing their diffusion into the surrounding cell matrix where chemical decomposition would take place. Fourth, to prevent compounds that might exert an inhibitory effect on the enzyme from reaching the active site. Fifth, to control and co-ordinate metabolic cross-talk that is mediated either by enzymes that function in different pathways or by intermediates that are shared between different metabolic pathways. And sixth, to provide a possibility for swift re-direction of metabolism by the formation of new metabolons that have altered polypeptide composition and product out-put, as might be demanded, for example, by environmental challenges. The advantages associated with the organization of a portion of or of an entire biosynthetic pathway in a metabolon are thus many-fold.

Metabolon formation typically involves specific interactions between several 'soluble' enzymes that might be anchored to a membrane either by membrane-bound structural proteins that serve as 'nucleation' sites for metabolon formation or by membrane-bound proteins, such as cytochrome P450s (CYPs), that directly catalyze one or more of the sequential channeled reactions carried out by the metabolon. Mounting evidence indicates that even pathways that were once thought to encompass only soluble enzymes are subject to subcellular structuring that involves metabolon formation. The focus of this review is metabolon formation and metabolic channeling in plant secondary metabolism.

Metabolons: stable, fragile or transient structures

Metabolons vary greatly in physical stability as determined, for example, by the strength by which the individual components are attached. Accordingly, the difference between an enzyme that is composed of multiple subunits that are present in a defined stoichiometric ratio and a metabolon is neither precise nor absolute. The phenomenon of channeling has been demonstrated experimentally in stable enzyme associations that exhibit

static channeling. Such enzyme complexes, which are exemplified by the bacterial tryptophan synthase, have dissociation constants that are so small that it is possible to determine the structure of the entire complex by X-ray crystallography [1]. A multifunctional protein that carries several active sites and catalyzes an entire biosynthetic reaction sequence, such as the Type I fatty acid synthases found in animals and fungi, might be the ultimate metabolon!

Metabolite channeling might also be achieved when the association between the polypeptide components of a metabolon is more dynamic. In such cases, unambiguous experimental documentation is much more difficult to acquire and is often met with great skepticism by the scientific community [2,3,4•]. The formation of some metabolons might be only transient. Transient complexes offer the possibility of fast exchange of some of the polypeptide components upon re-assembly, and thus provide a molecular mechanism to provide rapid fine-tuning or re-direction of metabolism. Metabolon formation might also be controlled by substrate binding. For example, a conformational change in cytochrome P450 enzymes results from substrate binding and mediates the attachment of cytochrome P450 reductase [5]. Numerous examples of transient enzyme complexes are known from primary metabolism (see [6••]), but their existence is very difficult to demonstrate.

Several mathematical models that simulate metabolic channeling and demonstrate the involvement of a metabolon in a specific pathway have been developed [6••]. The experimental data from which the parameters for such models are derived are often acquired from *in vitro* experiments, in which a dilute purified enzyme is administered to a single substrate. *In vivo*, the situation is very different and involves high protein concentrations, the presence of numerous different metabolites, metabolic cross-talk and the regulation of enzyme activities by a variety of mechanisms, including allosteric regulation and phosphorylation. Slow diffusion through the bulk solution might be circumvented by direct exchange of intermediates within metabolons, thereby dramatically increasing the workload of the enzymes involved. The assembly of polypeptides within a metabolon might introduce types of metabolic regulation that are different from those that regulate each of the free soluble enzymes, as has been observed for the regulation of polypeptides in a Calvin cycle metabolon that has thioredoxin and NADPH as effectors (see [7••]). The development of kinetic models that take such factors into account is a major challenge. Independent experimental evidence is necessary to assess the ability of mathematical models to truly reflect *in vivo* situations.

Classically, biological membranes such as the endoplasmic reticulum (ER) have been considered as homogenous fluid structures that are composed of lipid bilayers, which serve as a two-dimensional solvent phase for fully or partly embedded membrane proteins [8]. It is now clear that lipids can form micro-domains of unique biochemical composition. These domains are denoted 'rafts' and have been shown to constitute platforms for the assembly of metabolons [9]. The unique lipid composition of a raft is envisioned to direct proper anchoring and to facilitate and stabilize the subsequent assembly of the components required to form a specific metabolon. Rafts that are situated in the ER membrane system serve to organize the cellular location of the metabolons. In response to pathogen attack, for example, rafts might also convey the movement of these metabolons, guided by the actin cytoskeleton [10••], to other locations in the crowded cytoplasm. To afford optimal protection, rafts carrying metabolons that are involved in the synthesis of defense compounds against pathogens would profit from a cellular localization that is close to the pathogen entrance site, as this would facilitate the production of high local doses of defense compounds. Interestingly, rafts have also been shown to be preferred entrance points for pathogens [11].

Several excellent reviews are available on metabolic channeling and metabolon formation [7••,12,13,14•,15]. Strong evidence for the importance of metabolic channeling and the existence of metabolons has been acquired from studies of primary metabolism in microorganisms, animals and plants during the past 50 years. Well-known examples are tryptophan synthase, pyruvate dehydrogenase, glycine decarboxylase as well as the enzyme systems that catalyze the tricarboxylic acid cycle, Calvin cycle and glycolysis, fatty acid oxidation and the proteasome (see [7••]).

Metabolon formation and metabolic channeling in secondary metabolism

Plants produce an immense number of secondary metabolites, most of which have diverse and highly complex structures. A limited number of key genes encode the enzymes that are responsible for the synthesis of the pivotal backbone structures that constitute the hallmarks of the different classes of natural products, and great progress has been made in the identification of these genes (e.g. [16–23]). The subsequent decoration of the backbone structures generates the huge diversity of plant secondary products. The large majority of these decoration processes are mediated by a limited number of enzyme classes, such as glycosyl-, methyl- and acyltransferases, which are all encoded by multigene families. For example, the genome sequencing of *Arabidopsis* demonstrated that this plant species contains 112 UDP-glycosyltransferases ([24,25]; http://www.p450.kvl.dk/UGT.shtml). More than 200 different glycosides have been found in grapes [26]. Many of these downstream enzymes are regioselective or regiospecific rather than highly substrate specific [27,28•,29–31]. The positioning of enzymes

that have broad substrate specificity downstream of the conserved early pivotal enzymes of plant secondary metabolism opens the possibility of producing new secondary compounds without major re-structuring of the enzyme complement. Metabolic channeling and metabolon formation provide the key to resolving and avoiding potential negative interference in plant natural product formation either by narrowing substrate specificity as a result of conformational changes upon binding or because binding into the metabolon prevents access of unwanted substrates. A single glycosyl-, methyl- or acyltransferase might possess the ability to bind to different metabolons. In this manner, the possibility of combinatorially defined substrate specificity might explain how the desired substrate specificity is achieved with a minimum number of enzymes.

The possible role of metabolon formation in the synthesis of secondary plant products has been a point of discussion for many years, although the first evidence for the occurrence of metabolons in secondary metabolism was obtained three decades ago in the laboratories of Stafford [32] and Conn [33,34] in studies of the biosynthesis of phenylpropanoids and cyanogenic glucosides. Within recent decades, the experimental evidence has been greatly strengthened, especially by studies of flavonoid synthesis in Winkels laboratory (Figure 1; [35,36]). The existence of metabolons in several different pathways is now becoming increasingly apparent [36,37,38,39••,40•].

Research on metabolon formation and metabolic channeling has focused on the biosynthesis of natural products. However, metabolon formation could be just as important in catabolic pathways.

Isoprenoids

The site of synthesis varies for different classes of isoprenoids, with sterols and sesquiterpenes typically being synthesized in the cytosol, carotenoids, plastoquinone and phylloquinone in plastids, ubiqinone in mitochondria and rubber particles in specialized vacuoles [41]. Thus, several branches of the isoprenoid pathway lead to the synthesis and accumulation of structurally and functionally varied compounds in different cellular compartments. The reaction catalyzed by the membrane-bound enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is the first committed step in the mevalonate pathway of isoprenoid biosynthesis. All plants examined contain several isoforms of HMGR. In *Arabidopsis* cotyledons, some HMGR is located in the membrane system of the ER but a predominant portion is found within spherical vesicular structures that are located in the cytoplasm and within the central vacuole. Specific localizations might enable different isoforms of HMGR to channel intermediates into specific isoprenoid families. The cytoplasmic and intravacuolar vesicular structures appear to be derived from subdomains of the ER [15,42•]. The different types of HMGR-enriched vesicles might carry different sets of enzymatic activities, providing the physical basis for metabolic channels that each catalyze the synthesis of specific isoprenoid classes [42•].

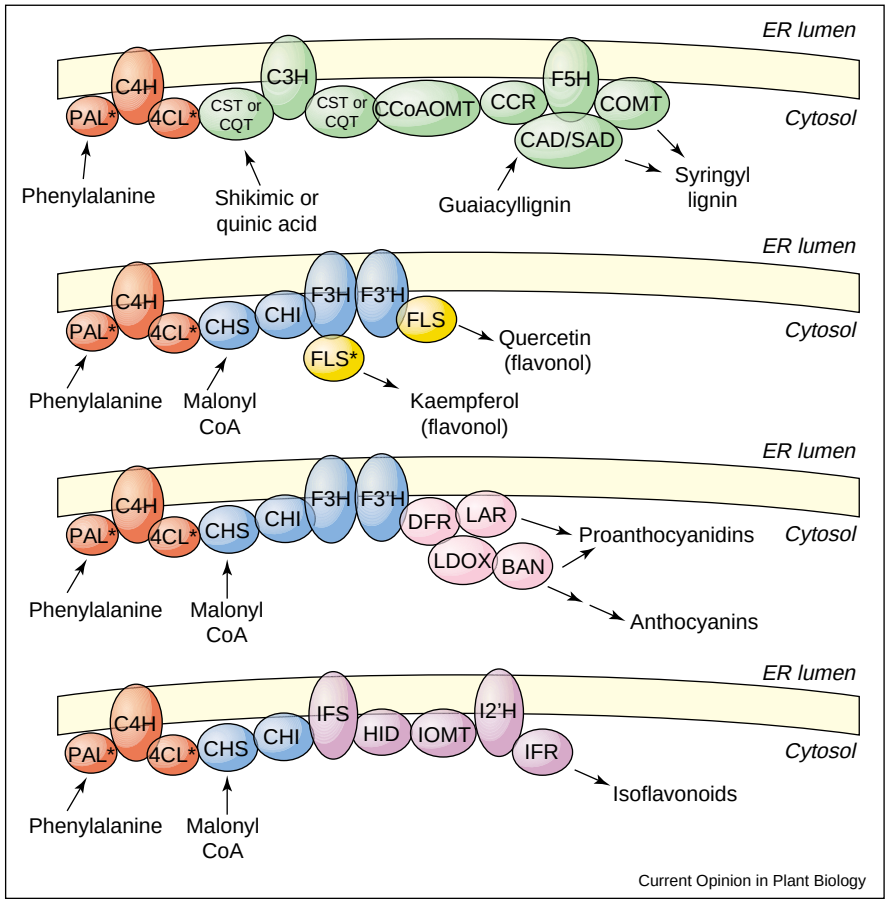
New knowledge of the gain and loss of fruit flavour compounds in wild (*Fragaria vesca*) and cultivated (*Fragaria ananassa*) strawberry has recently been provided [43••]. In cultivated strawberries, the terpenoid profile is dominated by the monoterpenoid linalool and the sesquiterpene nerolidol, whereas these are absent from wild strawberries in which they are replaced by olefinic monoterpenes and myrtenyl acetate (Figure 2). The gene that encodes nerolidol synthase (NES1), the enzyme responsible for linalool and nerolidol synthesis in cultivated strawberries was isolated and expressed in *Escherichia coli*. The NES1 enzyme, which is absent from wild species, generated linalool and/or nerolidol when supplied with geraniol diphosphate and/or farnesyl diphosphate, respectively. A comparison with NES sequences obtained from wild species revealed that the NES1 protein is truncated at its amino-terminal, resulting in lack of the amino-terminal transit peptide that directs plastid import. Accordingly, NES1 accumulates in the cytosol, where it is exposed to a new and larger pool of substrates, resulting in the effective synthesis of linalool and nerolidol. In cultivated strawberry, the gene encoding pinene synthase (PINS) contains a premature translation stop codon, which is caused by a frame-shift mutation, and this results in the absence of α-pinene and β-myrcene, and the downstream products myrtenol and myrtenyl acetate [43••]. These studies clearly illustrate how cellular compartmentation is important in defining natural-product profiles.

Alkaloids

Alkaloid structures vary from quite simple to highly complex. There is strong evidence that the initial steps in polyamine synthesis are organized in a metabolon [44]. Initially, the diamine putrescine is formed by the action of ornithine decarboxylase. Spermidine is then derived from putrescine by the action of spermidine synthase (SPDS). The final product, spermine, is formed by yet another aminopropyl-transfer reaction, which is catalyzed by spermine synthase. *Arabidopsis* contains two isoforms of spermidine synthase (SPDS1 and SPDS2) and a spermine synthase (SPMS) that shows sequence identity to SPDS1 and SPDS2. SPMS has been derived from a recent gene duplication of an ancestral SPMS-encoding gene. Heterodimers composed of SPDS1–SPDS2 and SPDS2–SPMS were formed in *Arabidopsis* as demonstrated by pairwise co-expression of epitope-tagged proteins and immunoprecipitation [44]. The protein complex had a mass in the range of 700 kDa, demonstrating that these aminopropyl transferases occur in metabolons *in vivo*.

The past decade has provided a lot of new molecular information on the biosynthesis of some of the most

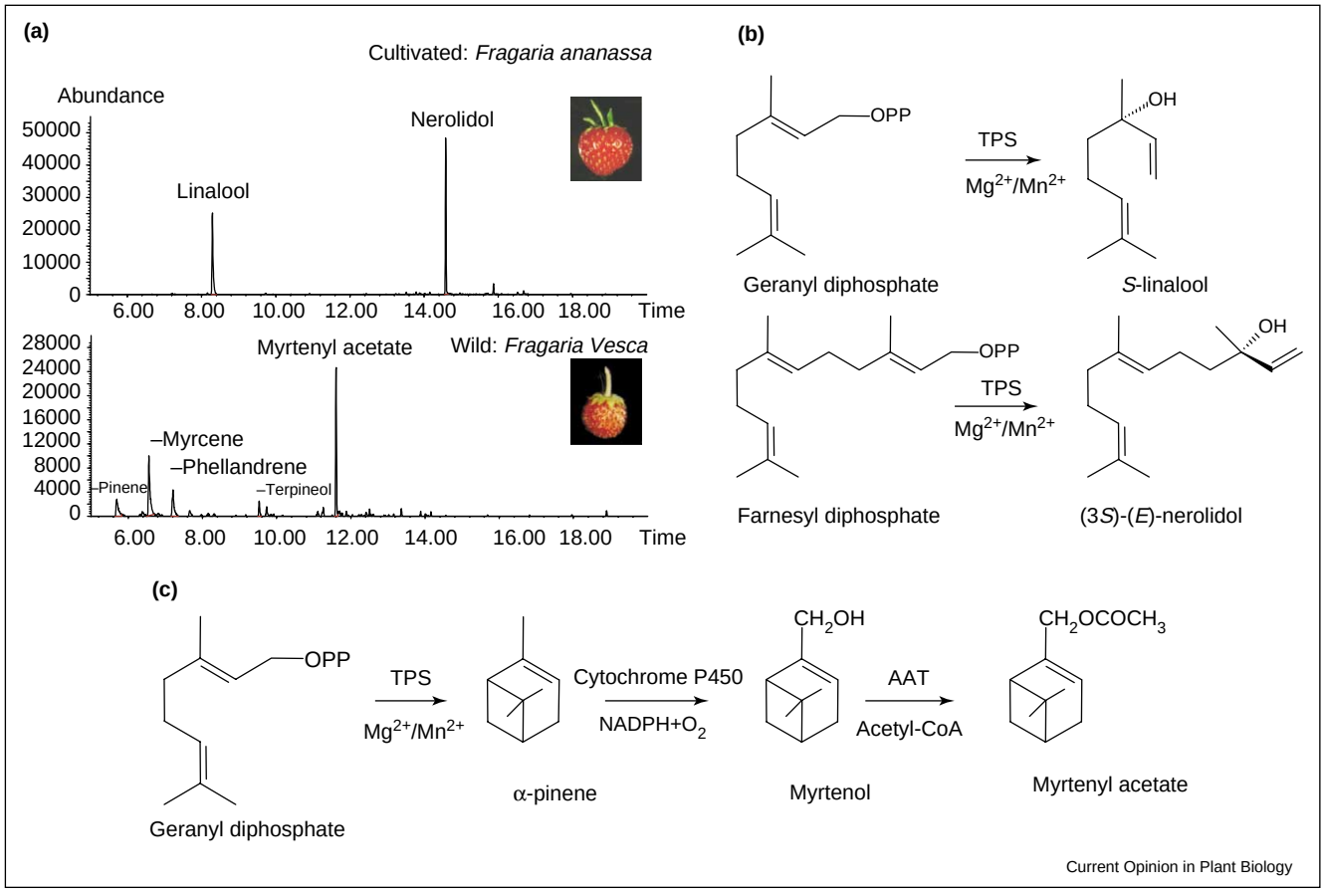
Figure 1



Organization of the branched pathways of phenylpropanoid metabolism within separate individual metabolons. Enzymes that participate in multiple branches are shown in red and blue, whereas enzymes that are thought to be unique to specific pathways are shown in other colours. Enzymes that are known to be present as multiple isoforms are marked with an asterix. The cytochrome P450 enzymes shown (C4H, *p*-coumaroyl-shikimate/quinate 3′-hydroxylase [C3H], coniferaldehyde (ferulate) 5-hydroxylase [F5H], flavonoid 3′-hydroxylase [F3′H], flavanone-3β-hydroxylase [F3H], IFS and isoflavone 2′-hydroxylase [I2′H]) are all membrane-spanning proteins, and are thought to provide nucleation sites or scaffolds that enable self-assembly of the soluble subunits. BAN, BANYULS (anthocyanidin reductase); CAD, cinnamyl-alcohol dehydrogenase; CCoAOMT, caffeoylCoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CQT, hydroxycinnamoyl-CoA D-quinate hydroxycinnamoyltransferase; CST, hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; IFR, 2-hydroxyisoflavanone synthase; IOMT, 2-hydroxyisoflavanone *O*-methyltransferase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; SAD, sinapylalcohol dehydrogenase. Based on the model of Winkel [7••].

complex alkaloids, including the description of all the individual steps in the conversion of tyrosine into berberine and the elucidation of most of the steps in morphine biosynthesis [45]. The alkaloid (*S*)-reticuline constitutes an important branch point in alkaloid synthesis because several different subclasses of isoquinoline alkaloids can be formed from this compound, depending on the subsequent regio- or stereospecific transformations that take place ([45]; Figure 3). Kutchan's laboratory has studied how these highly complex diversification processes are organized in the opium poppy (*Papaver somniferum*) [46••]. The cell-specific localization of five key enzymes in the metabolic grid has been determined in capsule, stem and root tissue. (*R,S*)-3'-Hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4'OMT) catalyzes the formation of (*S*)-reticuline and is a common precursor for both morphinan and tetrahydrobenzylisoquinoline alkaloids. The berberine bridge enzyme (BBE) was used as a specific marker for the sanguinarine pathway whereas (*R,S*)-reticuline 7-*O*-methyltransferase (7OMT) served as a marker for the laudanine pathway. Finally, salutaridinol 7-*O*-acetyltransferase (*Sal*AT) and codeinone reductase (COR) were used as markers for the morphine pathway (Figure 3). Immunolabeling studies in capsules demonstrated that the early stages of morphine synthesis are initiated by the action of tyrosine/dopa decarboxylase, resulting in the formation of the branch-point intermediate (*S*)-reticuline in parenchyma cells that

Figure 2



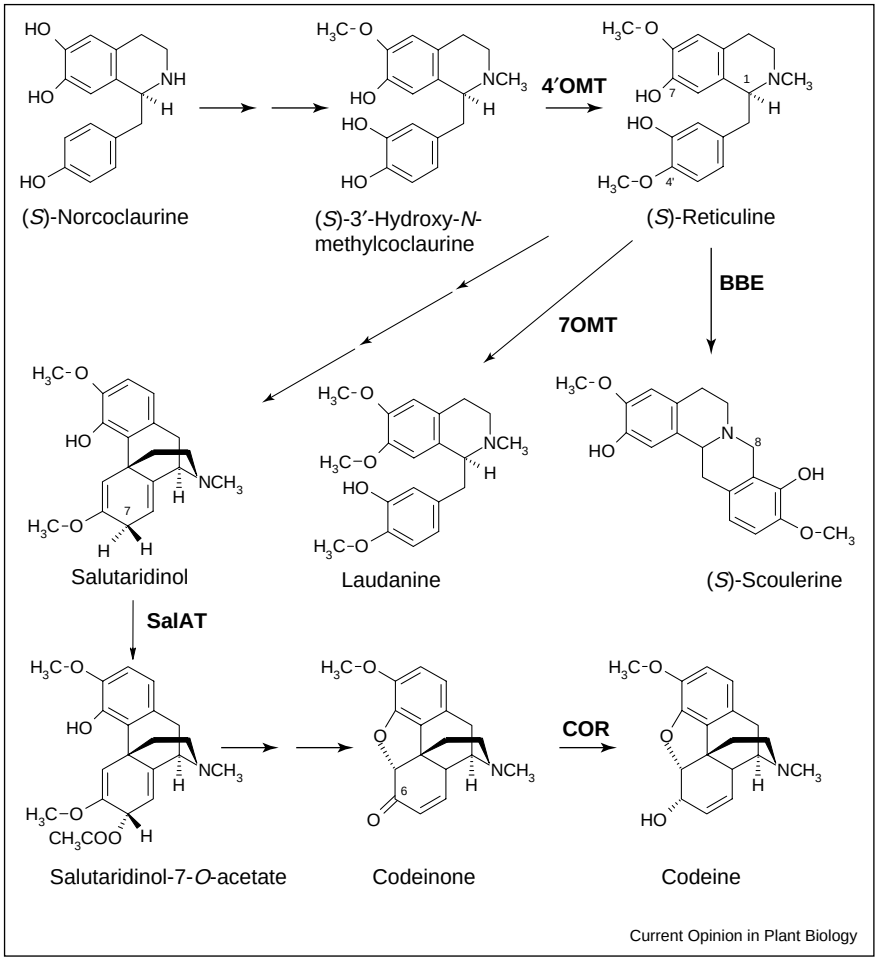
Terpenoid production in wild and cultivated strawberry. The terpenoids were detected by GC-MS headspace analysis of ripe fruits. Reactions that are catalyzed by terpene synthases (TPS) and acetyltransferases (AAT) forming linalool, nerolidol, α-pinene, myrtenol and myrtenol acetate are shown. Reprinted with permission from Aharoni *et al*. [43••].

are associated with laticifers. Diversification into laudanine formation takes place in parenchyma cells that are situated distal to the laticifers, whereas transformation of (*S*)-reticuline into morphine appears to be spatially located in parenchyma that surround the laticifers, at least up until the formation of salutaridinol 7-*O*-acetate. Downstream alkaloids such as thebaine and codeinone accumulate in vesicles in the latex, showing that at least one of the intermediates must be transported from the surrounding parenchyma cells to the laticifers. The notoriously high amounts of morphine and codeine that accumulate in laticifers in the opium poppy show that flux though a pathway can be efficient and high even though it depends on the transport of intermediates between different cell types. The separation of the intermediates in different cell types might help to avoid undesired secondary modifications (e.g. acetylations or methylations) of the alkaloids [46••]. Many of the *O*-methyl transferase sequences listed in the databases have been annotated as caffeic acid *O*-methyltransferases, although their *in vivo* homodimeric or heterodimeric complexes might show activity towards different classes of substrates, including alkaloids [28•,30,47]. Thus, any negative effects that result from the enhanced ability of enzymes that have been engineered by combinatorial biochemistry to catalyze secondary modifications of natural products might be counteracted by the spatial separation of the intermediates and interfering enzyme activities at the cellular level. An excellent example of channeling at the cellular level is provided by the different spatial organizations of the key enzymes in the metabolic enzyme grid that results in the synthesis of different morphinans and tetrahydrobenzylisoquinoline classes.

Phenylpropanoids

A plethora of different types of phenylpropanoids are produced from phenylalanine, with phenylalanine ammonia lyase (PAL) being the first committed enzyme in these pathways. In most plant species, PAL is encoded by small multigene families [48,49]. An important function of these different isoforms could be to channel intermediates towards the production of specific classes of

Figure 3



Schematic presentation of the biosynthesis of codeine, laudanine, and (*S*)-scoulerine from (*S*)-norcoclaurine in the opium poppy. The cellular localizations of the enzymes indicated have been determined experimentally. Based on the scheme of Kutchan and colleagues [46••].

desired phenylpropanoids. The evidence available to support spatial organization and channeling has recently been reviewed by Winkel [7••]. Additional evidence for the channelling of intermediates between specific isoforms of PAL and cinnamate 4-hydroxylase (C4H) has been provided by Dixon's laboratory [39••] using transgenic tobacco plants expressing epitope-tagged versions of two PAL isoforms (PAL1 and PAL*2*) and of C4H. Subcellular fractionation demonstrated that the PAL1 isoform is partly membrane bound and partly soluble, whereas PAL*2* appears to be restricted to the cytosol. Upon overexpression of C4H, both isoforms bind to the membrane fraction. This indicates that C4H might serve as the nucleation site for binding of the PAL isoforms and that PAL1 binds more strongly to C4H than does PAL*2* [39••]. Co-localization of PAL1 and PAL*2* with C4H has been demonstrated by dual-labeling immunofluorescence studies [39••]. Distinct isoforms of PAL have previously been assigned main functions in proanthocyanidin and lignin biosynthesis in quaking aspen (*Populus tremuloides*) [50]. 4-Coumarate:CoA ligase (4CL), a subsequent key enzyme in phenylpropanoid synthesis, also occurs as different isoforms. In *Arabidopsis*, these isoforms have been shown to possess different substrate preferences and expression patterns [51], with one isoform assigned a main function in lignin synthesis and a second in flavonoid synthesis [51]. Thus, most of the enzymes in phenylpropanoid metabolism appear to be organized in different metabolons that might be differentiated by the isoform (e.g. of PAL or 4CL) present, and by the nature of the participating downstream enzymes. A schematic illustration of the composition of metabolons envisioned in phenylpropanoid metabolism is shown in Figure 1.

The enigmatic 3-hydroxylation step in monolignol biosynthesis has recently been demonstrated to proceed after the initial conversion of the *p*-coumaric SCoA ester into shikimate, quinate or other esters of *p*-coumaric acid [52]. This discovery has introduced yet another layer of

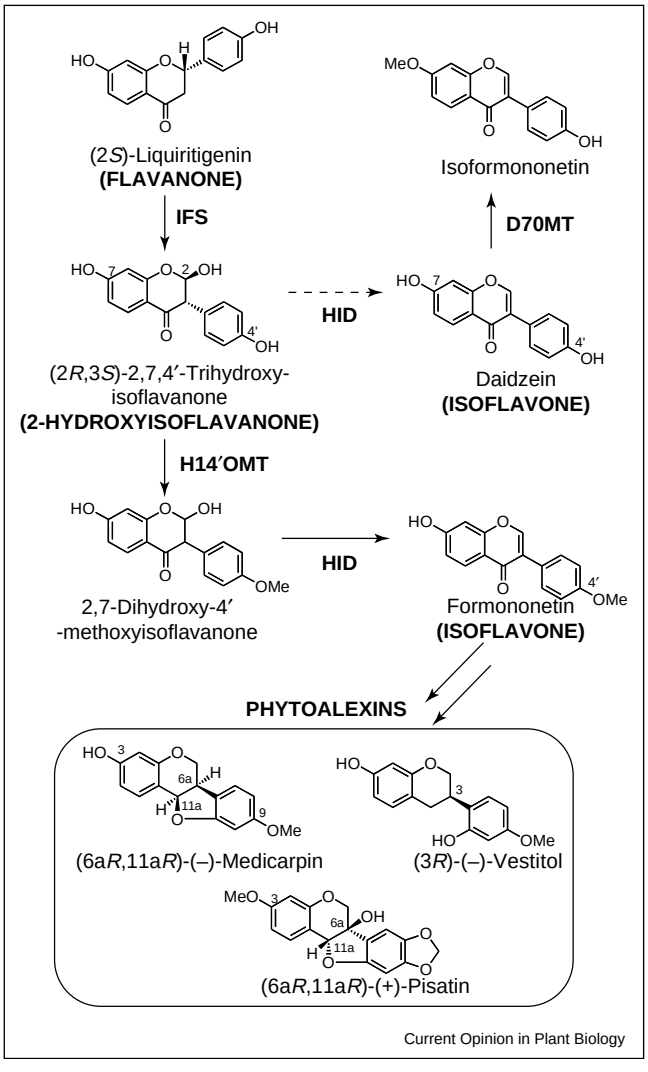
complexity to monolignol synthesis and might reflect the necessity to channel intermediates towards lignan and lignin formation along different branches, as supposedly facilitated by metabolon involvement.

Flavonoids and isoflavonoids

In terms of structure, flavonoids are based on the anthocyanidin chromophore but are divided into different structural classes on the basis of the oxidation state of their heterocyclic central pyran ring. As for other classes of natural products, the extensive structural divergence of flavonoids resides in the secondary decorations of the core chromophores [35,53]. Studies on channelling and metabolon formation in synthesis of secondary plant products have gained momentum within the past few years largely thanks to studies in Winkel's research group on flavonoid synthesis [7••,36]. This group has obtained strong support for specific interactions among at least one cytochrome P450 enzyme and several soluble enzymes by the combined use of a suite of technologies, including the yeast two-hybrid system, affinity chromatography, co-immunoprecipitation and immunolocalization [38,54].

Isoflavonoids are primarily found in leguminous plants. As for the flavonoids, the molecular diversity of isoflavonoids arises from secondary decorations of the basic isoflavone skeleton. The isoflavonoid skeleton is derived from a 4′-hydroxylated flavanone through the action of a 2-hydroxyisoflavanone synthase (IFS) that catalyzes a hydroxylation at the C-2 position accompanied by 1,2-aryl migration. The isoflavone is then formed by a dehydration reaction (Figure 4). In alfalfa (*Medicago sativa*), methylation of the 4′-hydroxy group to form important isoflavonoids, such as medicarpin and pisatin, has been argued to proceed in a metabolon either as an integrated part of the aryl migration reaction or perhaps in a reaction

Figure 4



Biosynthesis of *O*-methylated isoflavonoids in licorice. IOMT, 2-hydroxyisoflavanone *O*-methyltransferase. Based on scheme by Akashi *et al*. [56••].

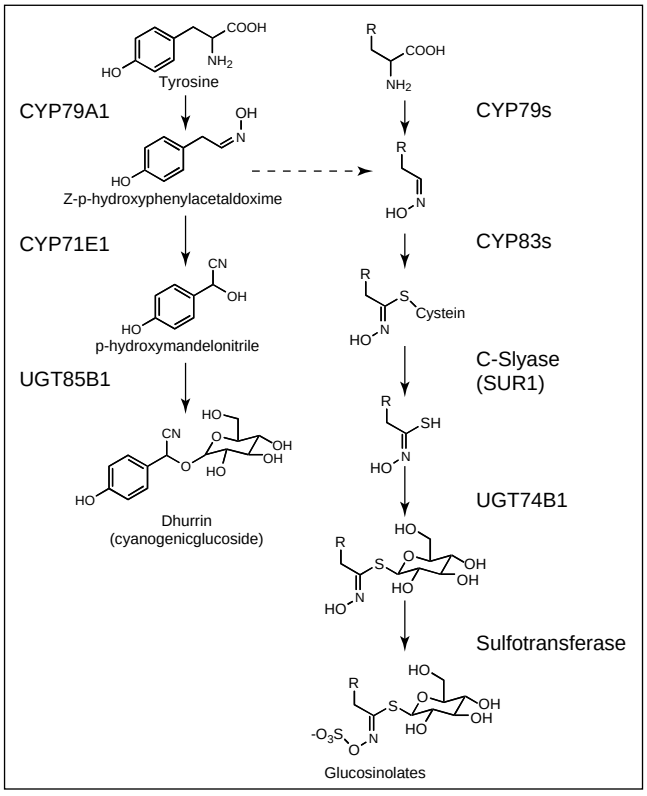
that involves a yet-unidentified transient intermediate. Alternatively, the binding of daidzein 7-*O*-methyltransferase (D7OMT) into the metabolon was hypothesized to change its specificity from a 7-*O*-methyltransferase into a 4′-methyltransferase [55]. This hypothesis has subsequently been challenged by the isolation of a cDNA clone from liquorice (*Glycyrrhiza echinata*) and *Lotus japonicus* that encodes a 2,7,4′-trihydroxyisoflavone 4′-*O*-methyltransferase (HI4′OMT), and by the demonstration of the presence of homologous cDNAs in other legumes [56••]. A 2-hydroxyisoflavanone dehydratase (HID) that converts 2-hydroxyisoflavanone into isoflavone has recently been isolated and shown to belong to the large carboxyesterase family [57]. This is yet another interesting example of the recruitment of an enzyme from primary metabolism to a function in secondary metabolism. The metabolon association of HI4′OMT and of HID remains to be investigated.

Cyanogenic glucosides

The biosynthesis of the tyrosine-derived cyanogenic glucoside dhurrin in sorghum (*Sorghum bicolour*) is catalyzed by two multifunctional cytochromes P450, denoted CYP79A1 and CYP71E1, and by a Family 1 UDP-glucosyltransferase, denoted UGT85B1 [16,19,27,40•,58]. *In vitro* experiments involving the simultaneous administration of dual radiolabeled intermediates demonstrated that the dhurrin pathway is highly channeled [33,34]. Transgenic plants expressing fusion proteins between the biosynthetic enzymes and spectral variants of green fluorescent protein, which were monitored by confocal laser scanning microscopy (CLSM), demonstrated that a metabolon that involves these enzymes is indeed formed and located in distinct domains of the ER [7••,40•]. The evolution of a metabolon for dhurrin synthesis would appear to be essential to ensure rapid conversion of the toxic *p*-hydroxymandelonitrile intermediate by UGT85B1 to prevent its dissociation into hydrogen cyanide and *p*-hydroxybenzaldehyde. The mechanism that enables the binding of UGT85B1 into a metabolon is now being investigated by molecular modeling and site-directed mutagenesis (K Thorsøe *et al.*, pers. comm.).

Transfer of the entire high-flux pathway for dhurrin formation from sorghum to *Arabidopsis* by genetic engineering proceeded essentially without inadvertent effects on the metabolome and transcriptome ([59•]; Figures 5 and 6). This demonstrates that effective encapsulation of toxic intermediates by metabolon formation is also achievable after heterologous expression in a plant species that would not naturally produce the same class of natural product [58,59•]. The insertion of an incomplete pathway (i.e. of CYP79A1 and CYP71E1) resulted in stunted plants, transcriptome alterations, the accumulation of numerous new glucosides derived from detoxification of intermediates in the dhurrin pathway, and the loss of the Brassicaceae-specific UV protectants sinapoyl glucose and sinapoyl malate [58,59•]. When introduced separately into *Arabidopsis*, CYP79A1 was able to establish highly efficient interaction with downstream glucosinolate-producing enzymes, presumably by participating in a new metabolon that resulted in the accumulation of large amounts of *p*-hydroxybenzylglucosinolate in *Arabidopsis* [60] and thereby changing the overall glucosinolate profile of *Arabidopsis* ([59•,61]; Figure 5).

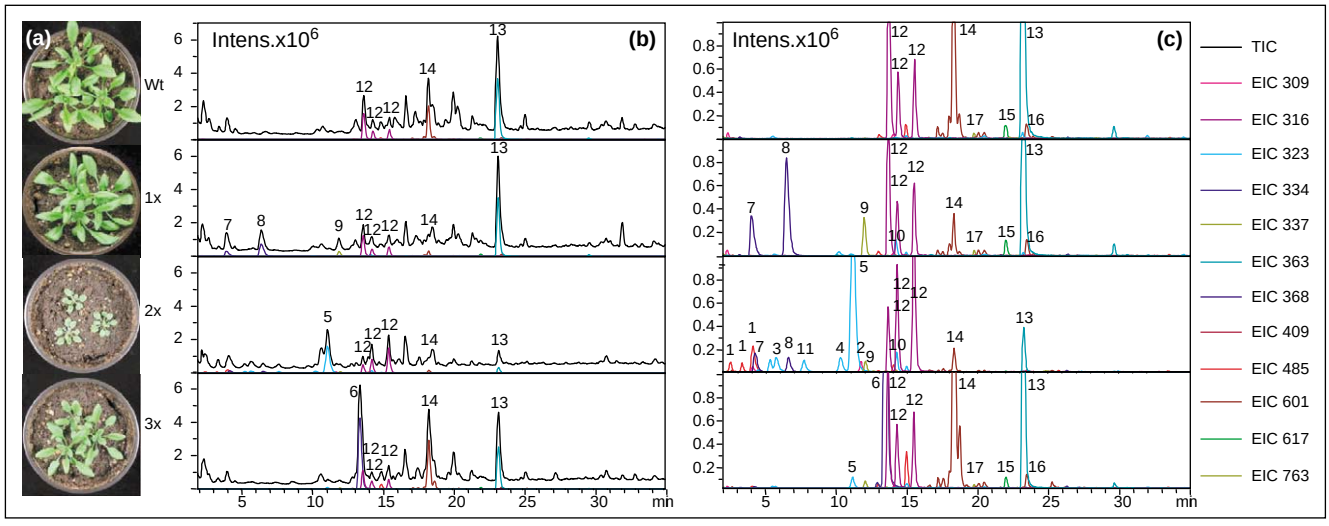
Figure 5



Biosynthesis of glucosinolates in transgenic *Arabidopsis* into which the sorghum-derived pathway for the synthesis of the cyanogenic glucoside dhurrin has been inserted. The dashed arrow indicates metabolic crosstalk. Reprinted with permission from Kristensen *et al.* [59•]. SUR1, SUPERROOT1.

The possibility of redirecting L-tyrosine into the glucosinolate or cyanogenic glucoside pathways without loss of plant fitness [58,62] demonstrates the existence of immanent routes for the transport and storage of new classes of natural products that are introduced into plants by genetic engineering, and an inherent ability to redirect and optimise the flux of intermediates to counteract imbalances in primary and secondary metabolism [40•,63]. The ability to accommodate altered levels of intermediates depends, however, on the type of metabolic cross-talk introduced. In *Arabidopsis*, tryptophan-derived oximes are key intermediates in both the formation of the phytohormone indole acetic acid and the synthesis of glucosinolates. CYP83A1 and CYP83B1 are the enzymes responsible for

Figure 6



Comparison of plant morphology and metabolite composition in wildtype *Arabidopsis* and transgenic *Arabidopsis* lines expressing the entire pathway for dhurrin synthesis or parts of it. Plants expressing sorghum CYP79A1 are designated 1x, plants expressing CYP79A1 plus CYP71E1 are designated 2x, whereas plants expressing the entire pathway (CYP79A1, CYP71E1 and UGT85B1) are designated 3x. **(a)** Morphological phenotypes and **(b)** metabolite profiles as monitored by (i) total ion current (TIC) traces and (ii) extracted ion chromatograms (EIC). 1: *p*-glucosyloxy benzylglucose; 2: *p*-glucosyloxy phenylmethanol; 3: *p*-glucosyloxy benzoid acid; 4: *p*-glucosyloxy phenylethanol; 5: *p*-hydroxybenzoyl glucose; 6: dhurrin; 7: *p*-hydroxybenzylglucosinolate; 8: desulfo *p*-hydroxybenzylglucosinolate; 13: sinapoyl malate; 14: sinapoyl glucose; 15: kaempferol-3-*O*-glucoside-7-*O*-rhamnoside; 16: kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside; 17: kaempferol-3-*O*-[rhamnosyl(1-2)glucoside]-7-*O*-rhamnoside; 9–12: unknown compounds. Reprinted with permission from Kristensen *et al*. [59•].

converting oximes into thiohydroximates [20,23,63,64] and UGT74B1 [65] catalyzes conversion of thiohydroximates into desulfoglucosinolates. Knockout of these enzyme activities disturbs auxin homeostasis and results in phenotypes that are reminiscent of those caused by auxin overproduction. Thus, channelling of tryptophan-derived intermediates into natural-product synthesis might serve an important function in balancing primary metabolism [20,23,64]. Surprisingly, disturbance of oxime metabolism affects phenylpropanoid metabolism and the monomer composition of lignin [66]. L-tryptophan accumulation that results from blockage of natural-product synthesis is also possible, as observed in a double-knockout mutant in *Arabidopsis* that lacks the tryptophan-metabolizing CYP79B2 and CYP79B3 enzymes [67]. Such plants completely lack indole-derived glucosinolates. Accordingly, the accumulation of free L-tryptophan does not necessarily disturb auxin homeostasis. Recently, the Myb transcription factor ALTERED TRYPTOPHAN REGULATION (ATR1) has been shown to control indole glucosinolate homeostasis by regulating the transcript level of tryptophan-synthesis genes and of CYP79B2/CYP79B3 and CYP83B1 of indole glucosinolate synthesis [68•]. Most probably, ATR1 will also turn out to regulate transcript levels of UGT74B1.

Conclusions and perspectives

The targeted introduction of new traits by genetic engineering, made possible by solid biochemical knowledge of metabolic channelling and metabolon formation, offers the opportunity to generate cultivars that have improved nutritional, health beneficial and agronomic properties that strictly adhere to the principle of substantial equivalence (i.e. the food is to be regarded as being as safe as its conventional counterpart and safety assessments should focus on the introduced defined differences). It seems most likely that the synthesis of the basic structures of natural products is facilitated by metabolon formation. Depending on cell type, developmental stage and the presence of abiotic or biotic stresses, additional enzymes that have broad substrate specificity might attach to the basic metabolons to prevent general access to their active sites and to secure desired specific modifications. In this manner, the cell is able to maintain the potential to specifically decorate a large array of natural products without having to produce a separate enzyme for each reaction. At the same time, this arrangement provides a reservoir of additional activities that might easily be selected for if they constitute an evolutionary advantage. Combinatorial chemistry is now widely used in chemical synthesis. But plants invented this approach millions of years ago to assure growth, development and survival in environments that impose many challenges.

Fortunately, a range of new advanced technologies are available for the study of metabolon formation. Modern forms of microscopy, such as confocal laser scanning microscopy (CLSM), might enable the direct imaging of meta-

bolons upon the stable or transient expression of target proteins as fluorescent-fusion proteins or as immunologically tagged proteins [39••,40•]. Direct visualization of metabolons, allowing assessment of their formation by sequential incorporation of component polypeptides, can now be achieved by atomic force microscopy [69]. Molecular approaches that are based on two-hybrid systems [38,44] or co-immunoprecipitation of epitope-tagged proteins to identify polypeptide components of metabolons has provided a wealth of new information [38]. New methodologies such as the use of Blue native gel electrophoresis (BN-PAGE) enable the isolation and characterization of metabolons in which adherence between the different subunits is weak [70]. Another effective isolation procedure is tandem affinity purification [71]. Subsequently, metabolon mass and polypeptide composition can be assessed by mass spectrometry [72]. In combination with surface plasmon resonance spectroscopy, these methodologies offer the unique possibility of determining the association and dissociation constants of the complexes. Such studies might be expanded further by the reconstitution of metabolons in nano discs [73,74]. The role of microtubules in the metabolic channelling of plant secondary metabolism is becoming more and more clear. Proteomic approaches to identify tubulin-binding proteins that have been purified by tubulin affinity chromatography and micro-tubule cosedimentation assays have now been established [10••] and will facilitate studies within this area.

Unraveling the mechanisms that control metabolon formation in natural-product synthesis will dramatically increase the potential of targeted metabolic engineering. If we meet this scientific challenge, we will have devised routes to enable the effective production of valuable medicinal drugs in plants and to re-model the crop plants of the future. We are convinced that the many dedicated scientists that work within this area will meet these challenges, and that some of the most spectacular scientific discoveries within the next ten years are going to be within natural-product synthesis in plants.

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