ELSEVIER

A role for intra- and intercellular translocation in natural product biosynthesis

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The formation and storage of plant natural products such as phenylpropanoids, terpenoids and alkaloids are dynamic and complex processes that involve multiple subcellular compartments and cell types. Evidence is emerging to show that consecutive enzymes of phenylpropanoid and flavonoid biosynthesis are organized into macromolecular complexes that can be associated with endomembranes, that monoterpenoid biosynthetic enzymes are exclusively localized to highly specialized glandular trichome secretory cells and that complex monoterpenoid indole- and morphinan alkaloids require a combination of phloem parenchyma, laticifers and epidermal cells for their synthesis and storage. Highly ordered, protein-mediated processes that involve intra- and intercellular translocation need be considered when attempting to understand how a plant can regulate the formation and accumulation of complex but well-defined natural product profiles.

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Introduction

Since the early days of the discovery and characterization of the enzymes of natural product biosynthesis in higher plants, evidence has accumulated of interactions between individual enzymes within a single pathway. The now classic example of such an interaction occurs in the formation of the cyanogenic glucoside dhurrin in *Sorghum bicolor* [1]. Intermediates that are derived *in situ* directly from the amino acid L-tyrosine were found to be utilized more efficiently for cyanogenic glucoside synthesis than those supplied exogenously to enzyme preparations. This phenomenon was referred to as 'channeling' and was attributed to multienzyme complexes. Further support for this observation was provided by work on the salt-marsh grass *Triglochin maritima* in which the biosynthesis of the epimer of dhurrin, taxiphyllin, also exhibited the channeling of intermediates [2]. Thus, the first level of organization within plant natural product biosynthesis appeared to be the association of consecutive enzymes in a pathway.

As more and more enzymes of natural product biosynthesis have been identified over the years, our view of the spatial organization of this synthesis has become more complex. Although cell cultures have been established that can accumulate a range of natural products [3], there are multiple examples of the failure of plant cell cultures to produce the same spectrum of natural products as the parent plant. Some enzymes of a pathway can be identified in *in vitro* cultures while others are apparently absent. This led to the idea that various degrees of cellular differentiation are necessary for biosynthesis and accumulation of some natural products, such as selected terpenes and alkaloids, implying that more than one cell type might be involved in the transformation of the branch-point primary metabolite to the stored or secreted end-product.

Today, we are well on our way to understanding complete biosynthetic pathways of selected plant natural products at both the enzyme and gene levels. Many natural products of complex structure require 20–30 enzymes for their synthesis, and each synthetic pathway is branched into a network that leads to a multiplicity of related chemical structures. There is a need to address the intra- and intercellular organization of such elaborate enzyme systems to understand how their highly branched pathways might be regulated. With increasing numbers of tools (in the form of genes and recombinant enzymes) at our disposal, we have begun to address the specialized organization of natural product pathways within cells and tissues using techniques such as *in situ* hybridization, immunocytochemical localization, two-hybrid assays and fluorescence energy resonance transfer (FRET). In this review, we present selected tantalizing examples of the potential intra- and intercellular trafficking of enzymes and natural product biosynthetic intermediates. Specifically, we review the localization and interactions of L-phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and later enzymes of phenylpropanoid metabolism in *Arabidopsis* and tobacco, the localization of several monoterpene biosynthetic enzymes in spearmint and peppermint, the localization of monoterpenoid indole alkaloid formation in Madagascar periwinkle and the localization of morphine synthesis in opium poppy.

Interaction of phenylpropanoid biosynthetic enzymes

The coordinated action of consecutive enzymes involved in the metabolism of phenylpropanoids was reported in the 1970s. In particular, an interaction between PAL and C4H was interpreted from biochemical experiments with microsomal preparations from cotyledons of cucumber (*Cucumis sativus*). In these preparations, there was a limited exchange of cinnamic acid, formed by membrane-associated PAL, with the free cinnamic acid pool that was added to the microsomal membrane system [4]. At the time, it was suggested that such a multienzyme complex model explains how the metabolic fate of L-phenylalanine, which can be metabolized into various phenolic substances (flavonoids, lignin, sinapate esters, stilbenes, tannins, anthocyanins and so on), could be determined early on at the amino-acid stage.

This enzyme complex model has been re-visited recently using very modern immunocytochemical localization, two-hybrid assays and FRET technology. The large number of natural products derived from L-phenylalanine and acetate via the phenylpropanoid pathway in any given plant dictates that subcellular organization should be crucial to many aspects of phenylpropanoid biosynthesis, such as local substrate concentration, regio- and stereospecificity and the sequestration of reactive biosynthetic intermediates. There are several groups of reactions through which channeling could occur [5]. Subcellular organization would also help to explain how certain modifying enzymes, such as methyltransferases or glucosyltransferases, can have such broad substrate spectra *in vitro* but result in the formation of only a few selected products *in vivo*. The flavonoid pathway in plants provides an ideal system with which to investigate enzyme complexes of secondary metabolism because several of the plants that produce flavonoids are genetically well-understood and amenable to manipulation.

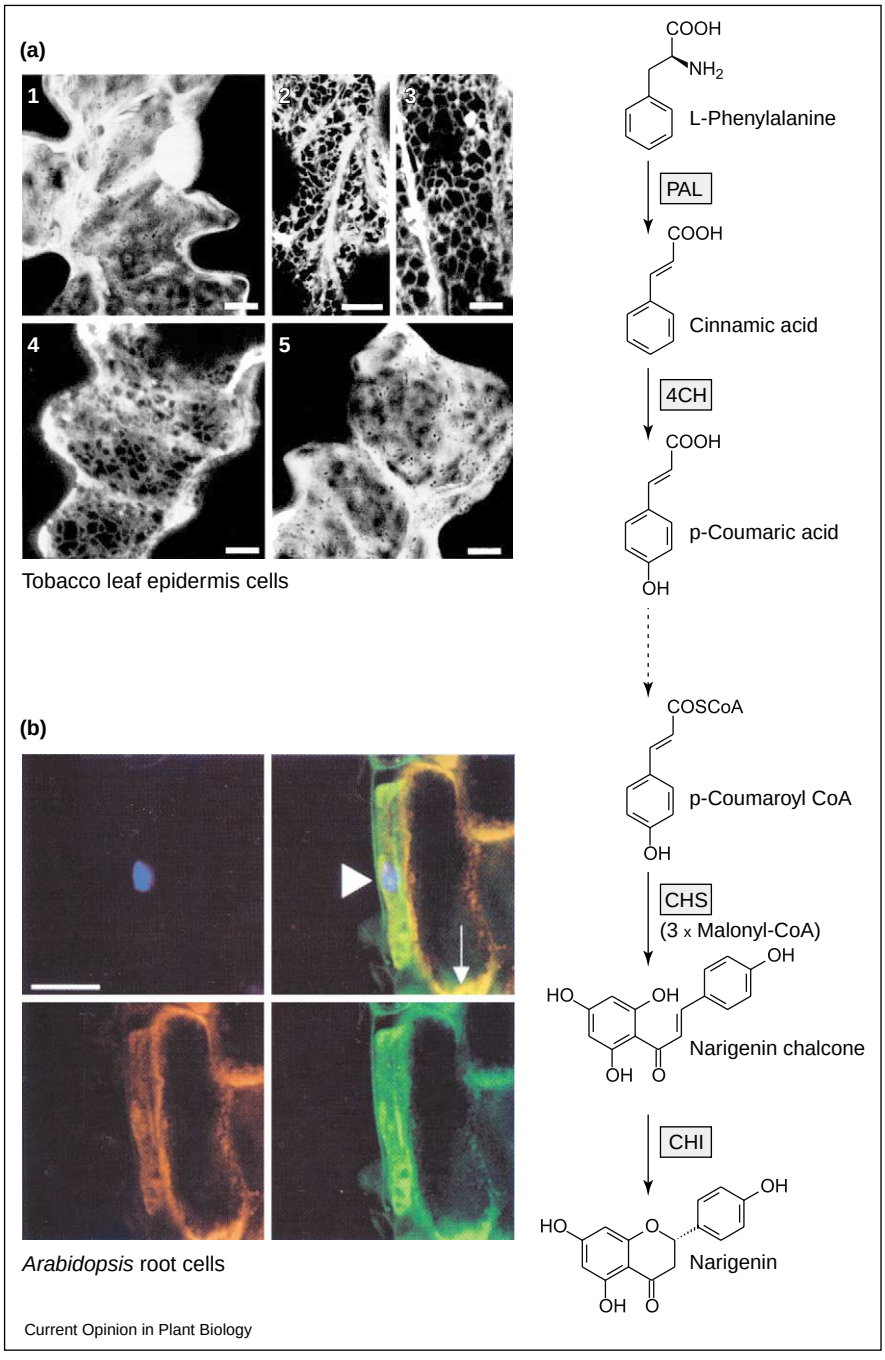
The interaction of two PAL isoforms (PAL1 and PAL2) with the cytochrome P-450-dependent monooxygenase C4H has recently been investigated in tobacco plants harboring epitope-tagged recombinant enzymes, in transient expression assays of PAL1-, PAL2-, and C4H-enhanced green fluorescent protein (eGFP) fusions and by FRET. Cell-fractionation studies had shown that PAL1, but not PAL2, localized to the endoplasmic reticulum (ER) in tobacco cells [6]. To establish whether a direct physical interaction occurs between PAL and the ER-localized P-450 C4H, the subcellular localizations of PAL1 (microsomal and cytosolic), PAL2 (cytosolic) and C4H (microsomal) were determined by cellular fractionation followed by protein gel-blot analysis [7••]. Co-localization of PAL1–eGFP and C4H–eGFP to endomembrane was further demonstrated by transient expression in leaf epidermal cells (see Figure 1a). Until this point, the co-localization of consecutive enzymes of phenylpropanoid and flavonoid biosynthesis had been elegantly demonstrated [6,8,9], but a direct physical interaction *in vivo* had not been shown. Using FRET, a non-invasive procedure for monitoring the protein–protein interactions of protein molecules that have been labeled with fluorophores [8], a weak protein–protein interaction between PAL and C4H could be observed at the surface of the endomembrane [7••]. It appears that channeling in this system does not result from a tight physical reaction between two enzymes. In fact, PAL (like many other natural product biosynthetic enzymes) would be considered to be operationally soluble. However, even a loosely associated complex comprised of different enzyme isoforms could function to channel (or partition) L-phenylalanine into the various phenylpropanoid-biosynthetic branches within a cell.

The interaction of chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol 4-reductase (DFR) in *Arabidopsis* seedlings was shown using the yeast two-hybrid system, which assays for direct protein–protein association. This interaction appeared to be directional: CHS interacted with DFR, CHI with CHS and DFR with CHI [9]. These data suggested that the flavonoid enzymes might form a globular complex rather than a linear array. In the same study, CHS, CHI and flavonol 3-hydroxylase were co-immunoprecipitated by anti-CHI IgY antibodies, further demonstrating protein–protein interaction among these enzymes. To corroborate these interactions *in vivo*, fluorescence immunolocalization was used to show that CHS and CHI accumulate in overlapping patterns in the primary root of *Arabidopsis* (see Figure 1b;[10]). Using the greater resolution provided by immunogold labeling, CHS and CHI were found to be organized in specific subcellular regions (i.e. the rough endoplasmic reticulum and small vacuoles) of flavonoid-synthesizing root cells [10]. Not all of the CHS and CHI protein was organized into complexes. This finding allows for a dynamic aspect to the formation of enzyme complexes, which might allow responses to physiological cues.

Cellular organization of monoterpene biosynthetic enzymes

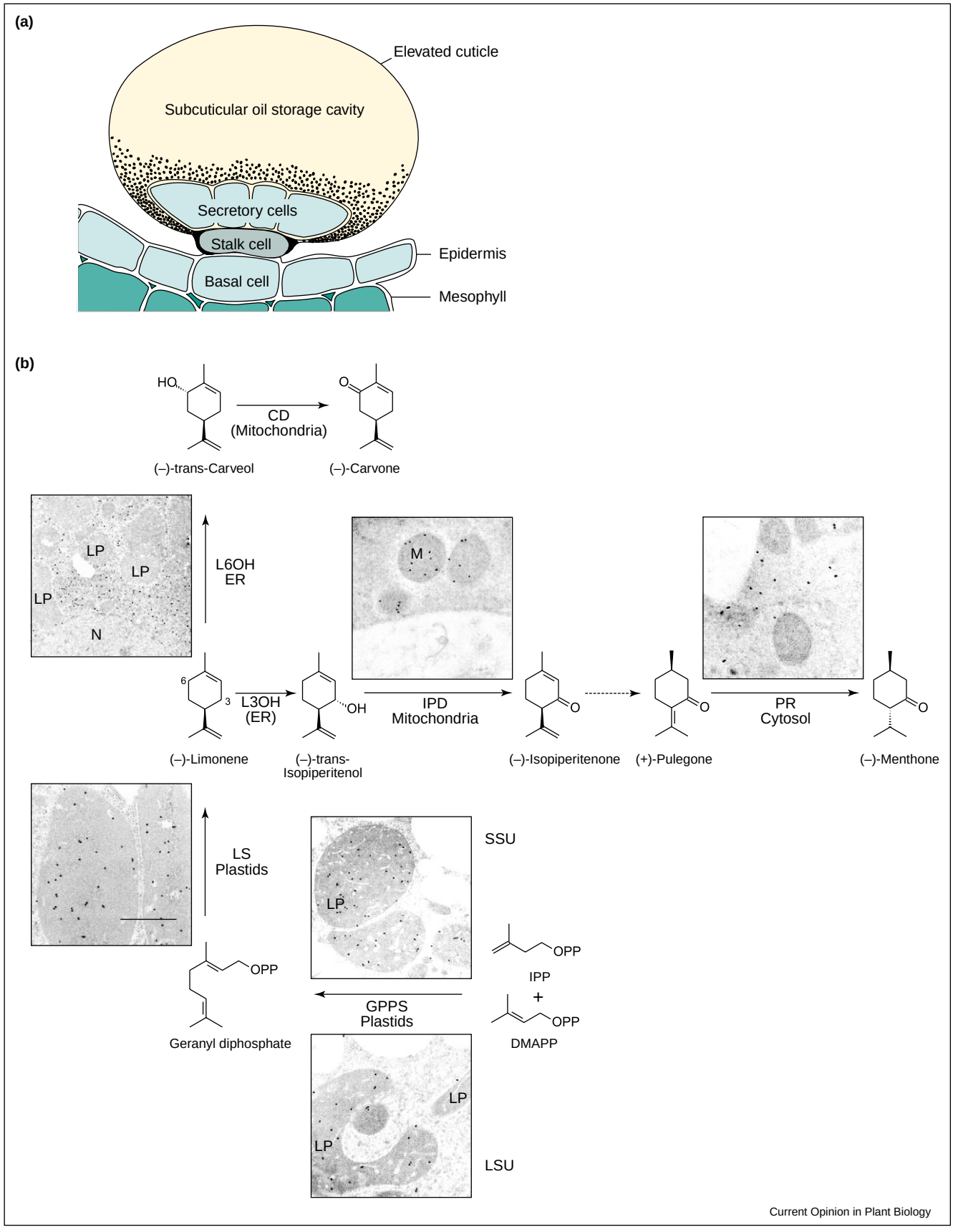
A plethora of plant species produce terpene-based resins and essential oils in specialized tissues, such as secretory cavities, ducts and glandular trichomes [11]. The knowledge of monoterpene biosynthesis provided by many years of investigation of mint species, including that of peppermint, is quite thorough [12]. Peppermint accumulates large quantities of monoterpenes in peltate glandular trichomes, which consist of one basal cell, one stalk cell, eight glandular secretory cells and a sub-cuticular oil storage cavity that is derived from the thick cuticle of the secretory cells (see Figure 2a; [13]). Isolated peppermint glandular trichomes were found to biosynthesize monoterpenes, defining the secretory cells as the site of mono-

Figure 1



(a) Localization of PAL– and C4H–eGFP fusion proteins in leaf epidermis cells of wildtype tobacco plants. Reproduced from [7••]. Expression constructs were introduced into the leaf tissue by particle bombardment and was fluorescence detected by confocal microscopy. (1) Free eGFP fluorescing throughout the cytoplasm and nucleus. (2) eGFP–HDEL (harboring a carboxy-terminal ER-retention signal) fluorescing in a reticulate pattern indicative of ER. (3) C4H–MA–eGFP (C4H membrane anchor [MA] fused to eGFP) showing the same reticulate pattern of fluorescence as eGFP–HDEL. (4) PAL1–eGFP showing reticulate and cytoplasmic fluorescence. (5) PAL2–eGFP showing cytoplasmic fluorescence. The scale bars represent 10 μm in (a) parts 1, 3, 4 and 5, and 20 μm in part 2. **(b)** Immunolocalization of CHS and CHI in wildtype *Arabidopsis* (Columbia) root cells by confocal laser scanning microscopy. Reproduced from [10]. Whole-mount seedlings double-labeled with anti-CHS and anti-CHI antibodies: CHI, red fluorescence; CHS, green fluorescence; nuclei pseudocolored blue [4′,6-diamidino-2-phenylindole (DAPI) stained]; co-localization of CHS and CHI is indicated by yellow color resulting from the merged red and green images. The arrowhead points towards a root epidermal cell containing CHS and CHI. The arrow points towards the co-localization of CHS and CHI at the apical end of a cortex cell. The scale bar represents 20 μm.

Figure 2


(a)
Elevated cuticle
Subcuticular oil storage cavity
Secretory cells
Stalk cell
Basal cell
Epidermis
Mesophyll
(b)
HO
CD
(Mitochondria)
O
(–)-trans-Carveol
(–)-Carvone
LP
LP
LP
N
L6OH
ER
M
6
3
L3OH
(ER)
OH
IPD
Mitochondria
O
O
PR
Cytosol
O
(–)-Limonene
(–)-trans-
Isopiperitenol
(–)-Isopiperitenone
(+)-Pulegone
(–)-Menthone
LS
Plastids
SSU
LP
OPP
IPP
+
OPP
GPPS
Plastids
OPP
DMAPP
Geranyl diphosphate
LP
LP
LSU
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terpene biosynthesis *in planta* [14,15]. Monoterpene biosynthesis begins with cyclization of geranyl diphosphate derived from isopentenyl diphosphate and dimethylally diphosphate, both of plastid origin [16]. The first dedicated enzyme of monoterpene biosynthesis in mint, (−)-limonene synthase, has been localized by immunogold labeling to the leucoplasts of peppermint oil gland secretory cells [17]. This was the only cell type within the glandular trichomes that was labeled by the anti-(−)-limonene synthase polyclonal antibodies. More recently, geranyl diphosphate synthase, (−)-limonene 6-hydroxylase, *trans*-isopiperitenol dehydrogenase and (+)-pulegone reductase have also been localized in mint species by immunocytochemical methods [18••]. All four of these enzymes were localized to the secretory cells of the pelate glandular trichomes of peppermint or spearmint (see Figure 2b). Immunogold labeling detected geranyl diphosphate synthase in the leucoplasts. The cytochrome P-450-dependent monooxygenase (−)-limonene 6-hydroxylase was associated with the endomembrane system, specifically with the ER. (−)-*Trans*-isopiperitenol dehydrogenase label was found in the mitochondria and (+)-pulegone reductase label in the cytoplasm. The localization of these five enzymes of monoterpene synthesis verifies that formation of this class of compounds is indeed restricted to the secretory cells of peltate glandular trichomes in mint and that multiple subcellular compartments are necessary for monoterpene biosynthesis.

The involvement of diverse organelles of secretory cells in monoterpene formation raises the question of whether intermediates are freely transported by diffusion, whether there is some kind of coordinated intracellular movement or whether the enzymes are organized into several macromolecular complexes that are localized to specific subcellular sites, as is thought to be the case for flavonoid biosynthesis [5]. A systematic attempt to functionally express a four-step plant monoterpene biosynthetic pathway in *Escherichia coli* resulted in the secretion of pathway intermediates into the medium. Even though all four enzymes were functionally present, the bacterial cell was presumably unable to properly traffic intermediates between enzymes [19]. Although little is understood about the mechanism of secretion from the secretory cells into the sub-cuticular oil storage cavity, an active transport is expected because of the unidirectional nature of secretion [15] and the large difference in the concentration of monoterpenes between the biosynthetic cells and the storage cavity. An additional level of complexity to be considered might be an interaction of biosynthetic enzymes with transporters (see chapter by Yazaki, this issue).

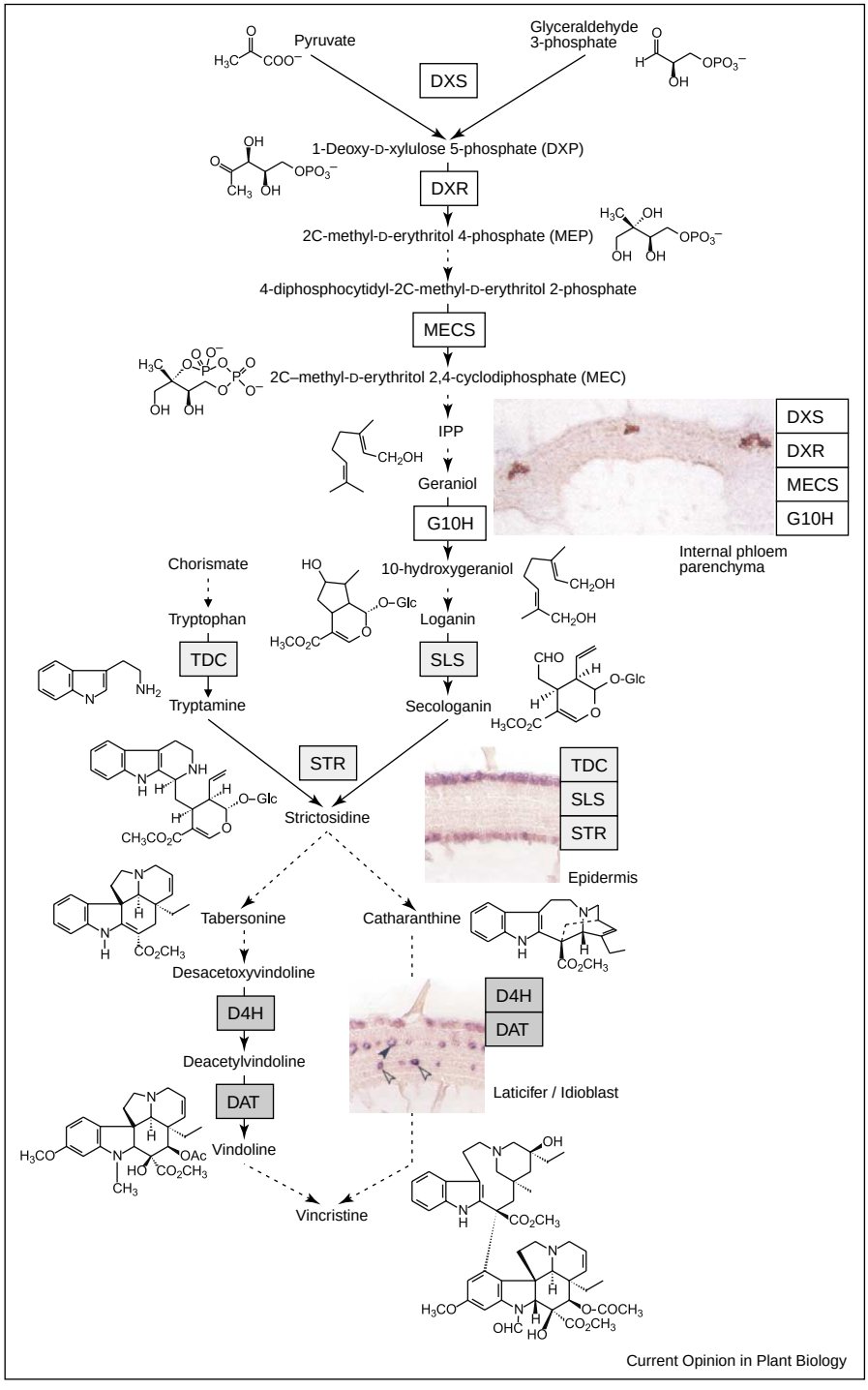
Multiple cell types of monoterpenoid indole- and benzylisoquinoline alkaloid biosynthesis

As for phenylpropanoids and monoterpenoids, selected classes of alkaloids accumulate in a tissue-specific manner. This distribution of many classes of natural products and their biosynthesis has become textbook knowledge [20,21]. In the alkaloid field, special attention has been paid to two particularly important pharmaceutical alkaloids, the chemotherapeutic dimeric monoterpenoid indole alkaloid vinblastine and the narcotic analgesic morphinan alkaloid morphine. Neither of these alkaloids has been successfully produced in plant cell culture, which suggested that cellular differentiation was essential to either their synthesis or their accumulation [22,23]. Given a selection of genes from the early and late stages (i.e. occurring either before or after branch-points in the pathways) of the biosynthesis of vinblastine in the Madagascar periwinkle (*Catharanthus roseus*) and morphine in the opium poppy (*Papaver somniferum*), the tissue-specific localization of the enzymes has now been investigated with immunocytochemical methods.

A series of three publications [24,25,26••] present results that suggest that at least three cell types are involved in the biosynthesis of the monoterpenoid indole alkaloid vindoline (a precursor to vinblastine) (see Figure 3). Biosynthesis of vindoline begins with geranyl diphosphate that is derived from isopentenyl diphosphate and dimethylally diphosphate of plastid origin [27,28] and with the decarboxylation of L-tryptophan [21]. The non-mevalonate pathway genes *DXS* (*1-deoxy-D-xylulose 5-phosphate synthase*), *DXR* (*1-deoxy-D-xylulose 5-phosphate reductoisomerase*) and *MECS* (*2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase*) were found by *in situ* hybridization to be expressed in internal phloem parenchyma of the young aerial organs of Madagascar periwinkle [26••]. The monoterpene geraniol that forms later is hydroxylated by geraniol 10-hydroxylase (G10H). The *G10H* transcript was localized to the same internal phloem parenchyma cell type as the non-mevalonate pathway genes [26••]. 10-hydroxgeraniol is converted to secologanin in a multi-step process. One of the enzymes of this conversion, secologanin synthase (SLS), was localized to the epidermal cells of developing leaves using immunohistochemistry and *in situ* hybridization [25]. The enzyme that decarboxylates L-tryptophan, tryptophan decarboxylase (TDC), and the enzyme that condenses the resultant tryptamine and

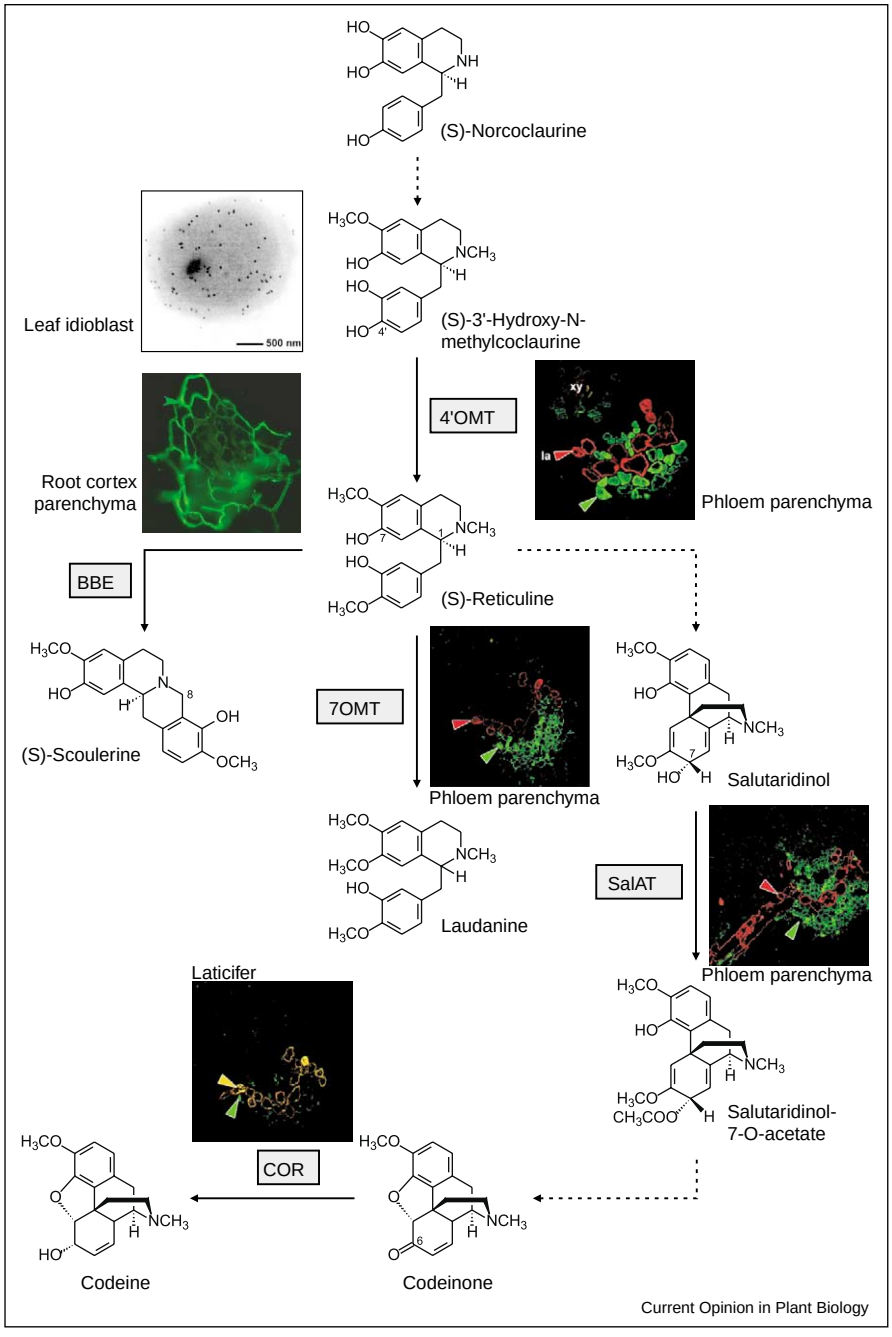
(Figure 2 Legend) Monoterpene biosynthesis in plants. **(a)** Schematic of a peltate glandular trichome of peppermint. Monoterpene biosynthetic enzymes have been biochemically and immunocytochemically localized to the secretory cells of the gland. **(b)** The monoterpene biosynthetic pathway in peppermint and spearmint. The enzymes indicated are the small-subunit (SSU) and large subunit (LSU) of geranyl diphosphate synthase (GPSS); (−)-limonene synthase (LS); (−)-limonene 3-hydroxylase (peppermint) (L3OH); (−)-limonene 6-hydroxylase (spearmint) (L3OH); (−)-*trans*-isopiperitenol dehydrogenase (peppermint) (IPD); (−)-*trans*-carveol dehydrogenase (CD); and (+)-pulegone reductase (PR). The localization of the enzymes of monoterpene biosynthesis in mint species, as revealed by immunogold cytochemistry is indicated by black spots. Data are from [17,18••]. LP, leucoplast; M, mitochondrion; N, nucleus.

Figure 3



Schematic of the biosynthetic pathway that leads to monoterpenoid indole alkaloids in Madagascar periwinkle. The three non-mevalonate (MEP) pathway genes *DXS*, *DXR* and *MECS*, and *G10H* are expressed in internal phloem parenchyma cells (image reproduced from [26••] with permission). Further along the biosynthetic pathway, the *TDC*, *SLS* and *STR* genes are expressed in epidermis and are directly involved in the formation of the central monoterpenoid indole alkaloid biosynthetic intermediate 3α(*S*)-strictosidine (image reproduced from [24] with permission). The *D4H* and *DAT* genes encode the final enzymes of vindoline biosynthesis and are expressed in laticifers and idioblasts (image reproduced from [24] with permission). Open arrowheads, idioblasts; closed arrowheads, laticifers.

Figure 4



Schematic of the biosynthetic pathway leading to tetrahydrobenzylisoquinoline-derived alkaloids in opium poppy. In the capsule and stem, 4′OMT, 7OMT and SalAT are found predominantly in phloem parenchyma cells and codeinone reductase is localized to laticifers. These are the site of morphinan alkaloid accumulation, as determined by fluorescence immunocytochemical localization (image reproduced from [29••] with permission). The berberine bridge enzyme has been localized to vesicles in idioblasts of young shoot (by immunogold labeling; data from [30]) and parenchyma cells of the root cortex (by fluorescence immunocytochemical labeling; image reproduced from [29••] with permission). Major latex proteins are represented by red arrowheads, biosynthetic enzymes by green arrowheads and the co-localization of major latex proteins and biosynthetic enzymes by yellow arrowheads. la, laticifer; MLP 15, major latex protein 15; Xy, xylem.

secologanin, strictosidine synthase (STR1), were also localized in the epidermal cells of leaves by immunohistochemistry and *in situ* hybridization [24]. By contrast, the later stages of vindoline biosynthesis, represented by the late-stage enzymes deacetylvindoline 4-*O*-acetyltransferase (DAT) and desacetoxyvindoline 4-hydroxylase (D4H), were associated with the laticifer and idioblast cells of leaves, as demonstrated again by immunohistochemistry

and *in situ* hybridization. Together, these results predict a translocation of pathway intermediates from internal phloem parenchyma to the epidermis and then on to laticifers and idioblasts. This is a tremendously dynamic process; however, we should also consider that this is a spatial picture of the biosynthesis of only relatively few of the two hundred indole alkaloids that are accumulated by Madagascar periwinkle.

Multiple cell types are also implicated in the biosynthesis of various benzylisoquinoline-derived alkaloids in opium poppy. Opium poppy accumulates more than eighty alkaloids that are derived from L-tyrosine, for which many biosynthetic enzymes and an increasing number of genes have been identified [21]. It has been known for almost two centuries that morphine accumulates in the latex of opium poppy. Previous biochemical data suggested the involvement of multiple cell types in alkaloid biosynthesis in poppy. The immunocytochemical localization of five enzymes of alkaloid formation in opium poppy has recently been reported. These enzymes are: (*R*,*S*)-3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT), which is central to the biosynthesis of tetrahydroisoquinoline-derived alkaloids; the berberine bridge enzyme (BBE) of the sanguinarine pathway; (*R*,*S*)-reticuline 7-*O*-methyltransferase (7OMT), which is specific to laudanosine formation; and salutaridinol 7-*O*-acetyltransferase (SalAT) and codeinone reductase (COR), which lead to morphine (see Figure 4; [29••]). In capsule and stem, 4′OMT, 7OMT and the SalAT were found predominantly in phloem parenchyma cells, whereas codeinone reductase was localized to laticifers, the site of morphinan alkaloid accumulation. In developing root tip, 4′OMT, 7OMT and SalAT were found in the pericycle of the stele, and BBE was localized to parenchyma cells of the root cortex [29••] and to idioblasts of leaf [30]. Tyrosine decarboxylase transcript has been localized by *in situ* hybridization to phloem cells in aerial plant parts of opium poppy [31], and so it appears that the early stages of morphine biosynthesis, starting with the decarboxylation of the amino acid L-tyrosine, occur in parenchyma cells surrounding laticifers. The later stages of morphine biosynthesis occur in the laticifer, which is the storage site of the morphinan alkaloids thebaine, codeine and morphine. As specialized cell types, such as phloem parenchyma and laticifers, are involved in both monoterpenoid indole- and morphinan alkaloid synthesis, it can be expected that these biosynthetic processes might be regulated by factors that are involved in cell differentiation.

Conclusions

Our view of plant natural product biosynthesis is becoming more and more refined into one of a highly organized, dynamic process. Whether biosynthetic intermediates are transported by diffusion or whether there is a type of coordinated intracellular movement with enzymes organized into macromolecular complexes should be carefully addressed. The involvement of diverse organelles in plant natural product biosynthesis dictates that intracellular translocation must occur, and this process need be investigated. Given the multiple cell types and extracellular spaces that are clearly involved in the synthesis and storage of phenylpropanoids, terpenoids and alkaloids, our attention should also be drawn to intercellular translocation, which is likely to involve active transport processes. The interaction of macromolecular biosynthetic enzyme complexes with metabolite transporters might also be a consideration. Envisioning highly ordered, protein-mediated processes, which in part occur in specialized cell types, is also one way to understand how a plant can regulate the formation and accumulation of complex but well-defined natural product profiles. The rational design of plants with improved natural product profiles will most likely also have to take the various levels of complexity of biosynthesis and storage into consideration.

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- •• of outstanding interest

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The work described in this article uses transient expression of GFP-fusion proteins to provide further evidence of the association of PAL1 and C4H with the ER in tobacco. PAL2 is found to have a cytosolic localization. Co-localization of PAL and C4H is confirmed by FRET, which suggests that the interaction between the two proteins is not tight.

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This article shows the bigger picture of monoterpene biosynthesis by using immunogold cytochemistry to localize four of the enzymes of monoterpene synthesis in mint. The results indicate that monoterpene synthesis is a dynamic process that involves multiple subcellular compartments within the secretory cells of mint glandular trichomes, which include plastids (which contain limonene synthase [LS]), ER (to which limonene 3-hydroxylase and limonene 6-hydroxylase localize), mitochondria (containing isopiperitenol dehydrogenase) and the cytosol (containing pulegone reductase). These results provide the basis for investigation of potential enzyme complexes or metabolite trafficking routes in mint species.

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