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Review

From waste products to ecochemicals: Fifty years research of plant secondary metabolism

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Abstract

The isolation of morphine (‘*principium somniferum*’) by Friedrich Wilhelm Sertürner about 200 years ago is generally accepted as the beginning of scientific phytochemistry (plant secondary products research). For about 150 years this research addressed almost exclusively the isolation and structure elucidation of new plant products. It had great impact on the development of modern organic chemistry and pharmaceutical industry and provided the chemical basis for biological research on plant secondary metabolism, which began about 50 years ago. The historical development of this field to its present state of knowledge will be considered in this review from three angles of vision: mechanistic, functional and evolutionary perspectives. Mechanistic research started on the metabolite level and was initiated by the availability of radioactive nuclides in the early 1950s. By means of sophisticated tracer techniques, the biosynthetic routs of most secondary pathways were outlined and provided the basis for the enzymatic characterization of biosynthetic pathways in the 1970s and 1980s, followed by the identification of the corresponding genes beginning in the late 1980s. During this 50-year period of intensive research a change of paradigm occurred addressing the question: why do plants synthesize this immense rich diversity of secondary metabolites comprising more than 200,000 structures? For a long time regarded as waste products or assigned with various other attributes their indispensable role as components of the survival strategy of plants in a mostly hostile environment appears now generally accepted. Along with the great progress in the field of chemical ecology, the emerging field of molecular evolution provided crucial evidence that during evolution of secondary metabolism genes encoding enzymes of plant’s primary metabolism were duplicated, recruited and diversified for new functions under the everlasting and continuously changing selection pressure of the environment.

Keywords: Secondary metabolism; Review; History; Biosynthetic pathways; Turnover and degradation; Cell-type specific localization; Ecological functions; Molecular evolution

Contents

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1. Introduction

The phenomenon of secondary metabolism and its differentiation from basic or primary metabolism was already recognized in the second half of the 19th century. In his textbook, published in 1873, Julius Sachs, one of the "founding fathers" of plant physiology, gave the following definition:

> "Als Nebenprodukte des Stoffwechsels kann man solche Stoffe bezeichnen, welche während des Stoffwechsels entstehen, aber keine weitere Verwendung für den Aufbau neuer Zellen finden. …. Irgend eine Bedeutung dieser Stoffe für die inner Ökonomie der Pflanze ist bis jetzt nicht bekannt". (Sachs, 1873, p. 641).
>
> "One can designate as by-products of metabolism such compounds which are formed during metabolism but which are no longer used in the formation of new cells. …. Any importance of these compounds for the inner economy of the plant is so far unknown".

This definition is still valid; we simply have to substitute the terms "by-products" by "secondary products" and "inner economy" by "primary metabolism". The differentiation between "secondary" and "primary" metabolism (and metabolites) was probably first introduced by Albrecht Kossel in 1891 (Mothes, 1980). "Secondary products" is synonymous to "natural products", a term more commonly used by chemists. Plant secondary metabolites include a vast array of compounds that to date sum up to more than 200,000 defined structures. Plant secondary metabolism covers all physiological and biochemical facets of "secondary products" including functional and evolutionary aspects. Research in this area began not before the middle of the 20th century (Fig. 1). At its beginning it was just a compilation of various classes of chemical constituents (e.g., terpenoids, phenylpropanoids, polyketides and alkaloids) and the description of their distribution within the plant kingdom and their occurrence and accumulation within plant tissues (Bonner, 1950; Peach, 1950).

The study of plant secondary compounds can be considered to have started in 1806 when Friedrich Wilhelm Sertürner isolated morphine ('*principium somniferum*') from opium poppy (Fig. 1). This first demonstration that the active principle of a plant drug can be isolated and attributed to a single chemical compound initiated natural product chemistry. In rapid sequence the isolation of one active principle after another followed. The speed at which this field developed, greatly influenced and directed major areas of organic chemistry, particularly synthetic, analytical and pharmaceutical chemistry. It also advanced the first foundations of pharmaceutical industry and drug research. The first synthesis of a secondary product, indigo, by von Baeyer in 1886, provided a milestone in synthetic organic chemistry. The chemical structure of morphine was elucidated in 1923, whereas the first total synthesis of its complex structure was not completed until 1950, almost 150 years after its isolation (Fig. 1). These few introductory remarks should just recall and emphasize the "prehistory" of plant secondary metabolism which started and stayed for a long time *Natural Product Chemistry* with all its

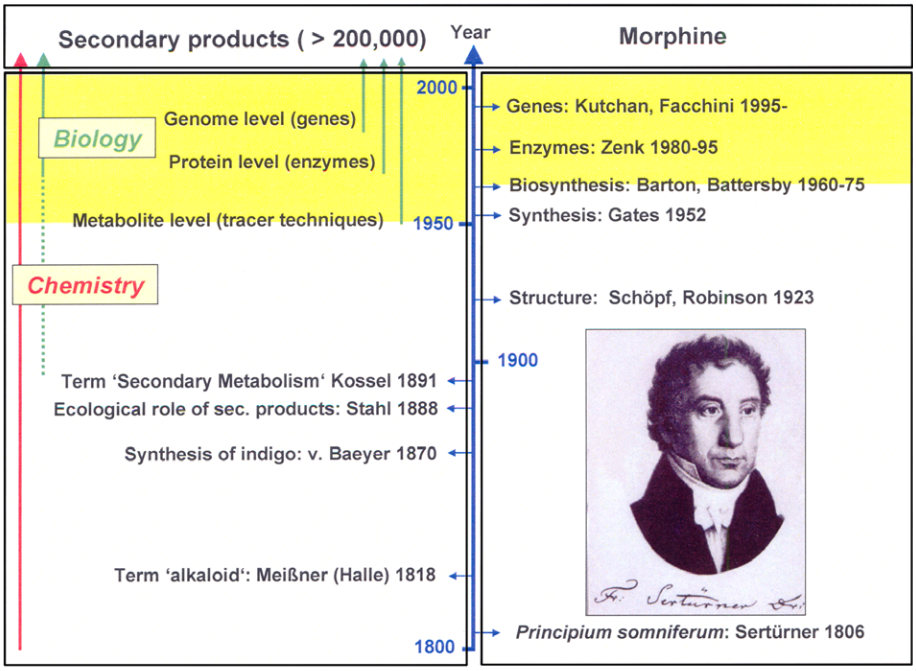

Fig. 1. The history of secondary product research started about 200 years ago with the first isolation of a natural product (*principium somniferum*, i.e. morphine) by Friedrich Wilhelm Sertürner. The left panel illustrates the general history the right panel the history of morphine with a few milestones. Research in 'secondary metabolism' is about 50 years old (shaded yellow). It began with the first biosynthetic studies by application of radioactively-labeled tracers.

facets. For about 150 years after the isolation of morphine, the plant was almost exclusively used as a profitable and inexhaustible source for novel natural products. First experiments addressing biosynthetic mechanism started not before the early 1950s (Fig. 1), when the first radioactively-labeled precursors became available.

It is not feasible to cover comprehensibly this vast field in a single review. Therefore, this survey describes the short but eventful history of plant secondary metabolism to its current status as the author's personal view with a major focus on its changeful functional perspectives. Due to its immense metabolic diversity the field of plant secondary metabolism has numerous scientifically important facets. There are thousands of outstanding contributions which demand quotation. I apologize to everybody I did not mention. The review is devoted to all who contributed to a better understanding of plant secondary metabolism.

2. Research developments

2.1. Mechanistic aspects

In the first half of the 20th century proposals about the formation of secondary metabolites in plants were based mainly on analogy with reactions of organic chemistry and on comparison of structures rather than on biochemical evidence (Robinson, 1981). This changed dramatically when tracer techniques with radioactively-labeled nuclides were introduced in the early 1950s. The availability of ^{14}C- or ^{3}H-labeled compounds enabled feeding studies that followed Melvin Calvin's pioneering work on the early steps of photosynthetic CO_2 fixation, which began already in 1945. In the following decades the biosynthetic pathways of almost all important classes of secondary metabolites were outlined. In all these studies, however, the plant remained a black box. The plant was only involved twice: at the beginning of the experiment for tracer application and days or even weeks later for extraction and analysis of the labeled products. The major innovation in these studies was a competent synthetic chemistry needed for the synthesis of the desired labeled precursor and the often ingenious 'degradation-chemistry' to identify the ^{14}C-labeled C-atoms in the products' carbon skeletons. In the 1970s improved MS and NMR techniques allowed an efficient application of precursors and intermediates labeled with stable nuclides (i.e., ^{2}H, ^{13}C, ^{18}O and ^{15}N). The great advantage of these methods is that chemical degradation is no longer necessary. The labeled atoms are allocated 'online' by molecular fragmentation and nuclear magnetic resonance. New MS methods advanced in sensitivity, combined with high resolution separation techniques like gas chromatography (GC–MS) and liquid chromatography (LC–MS), gave a fresh impetus to the chemical analysis of compounds (Marston, this issue). Quantitative ^{13}C NMR techniques are successfully applied in retro-biosynthetic approaches that allow tracing a particular ^{13}C atom of a distant precursor (e.g., glucose) to the product (Eisenreich and Bacher, this issue).

The outline of a biosynthetic pathway through tracer techniques is a mechanistic prerequisite for a detailed enzymatic characterization of this pathway (see below). An impressive example illustrating the importance of modern tracer techniques is the discovery of an alternative pathway of terpenoid biosynthesis, a pathway that despite long-known evidence was not discovered before the mid 1990s. The discovery of this pathway is also a descriptive example how a dogma may hamper, or even prevent, progress. Since the formulation of the "biogenetic isoprene rule" by Ruzicka (1953) and the subsequent elucidation of the mevalonate pathway leading to "active isoprene" (i.e., isopentenyldiphosphate, IPP and dimethylallyldiphosphate, DMAPP) it became a rule that in all organisms the "active isoprenes" are provided via the mevalonate pathway. Over the years terpenoid biosynthetic studies with labeled acetate or mevalonate in various organisms including plants mostly confirmed this concept. The only obvious exception is plant monoterpenoids, diterpenoids and carotenoids. Tracer studies in various labs with mevalonate yielded extremely low levels of tracer incorporation and early doubt about a universal role of mevalonate as terpenoid precursor arose, however without consequence, as the following quotation shows:

> "The observed incorporation of mevalonate into monoterpenes is so low that it might be well cited as evidence for an alternative pathway, if there were any positive evidence for such an alternate pathway". (Loomis, 1967 p. 64).

It took more than 25 years until an alternative route of the synthesis of active isoprene was detected in plants. Schwarz studied the incorporation of ^{13}C-labeled glucose into ginkgolide A, a diterpene of *Ginkgo biloba*, and found a labeling pattern that indicates the formation of "active isoprene" via a route, which to day is well known as the methylerythritolphosphate (MEP) pathway. This study was the first report on the MEP pathway in phototrophic eukaryotes, "hidden" in an exceptional thesis (Schwarz, 1994), and more easily accessible to the scientific community only five years later (Arigoni and Schwarz, 1999). Independently and a bit earlier, the MEP pathway has been detected in prokaryotes, in the biosynthesis of bacterial hopanoids (Rohmer et al., 1993). The plant MEP pathway and its restriction to plastids as the sites of the biosynthesis of mono-, di-, and tetraterpenoids as well as its bacterial counterpart were subject of detailed recent reviews (Rohmer, 1999; Eisenreich et al., 2004).

In addition to biogenetic studies, tracers were also successfully applied in early physiological studies. Already in the 1950s, tracers were applied to identify the organ-specific sites of synthesis of secondary compounds, to discover whether a compound is translocated within plants, e.g. via the phloem or xylem, and to get information about possible turnover or degradation of secondary metabolites (Mothes, 1960). At that time it was also demonstrated that, for instance, alkaloids may be synthesized in the roots (e.g., nicotine in tobacco and hyoscyamine in *Datura*, *Atropa* and *Hyoscyamus*) or in the shoots (e.g., quinolizidine alkaloids in lupines, coniine in hemlock and steroid alkaloids in potatoes). Such studies confirmed relatively early on that the formation of many secondary metabolites occurs well coordinated in time and space during the plant's ontogenesis (Wiermann, 1980).

A discouraging opinion could be heard frequently in the 1950s to the early 1960s: "It is almost impossible to isolate active enzymes involved in plant secondary metabolism". The acidic cell sap of plant vacuoles, often stuffed with tannins and other phenolic compounds, rapidly denatured active proteins. Preparation of acetone dry powders of plant materials as source for enzyme extraction and the addition of poylvinylpyrrolidone (Loomis and Battaile, 1966) and other polymeric absorbents during enzyme extraction were an advantage but still did not substantially improve the situation. At that time many reports about reactions in crude tissue homogenates and cell-free protein extracts appeared but purification of the responsible enzymes remained an exceptional success. As a pioneering work in the field, the purification and characterization of the core-enzyme of phenylpropanoid biosynthesis, phenylalanine ammonia-lyase (PAL), in Eric Conn's lab must be mentioned (Koukol and Conn, 1961). Further examples followed slowly, but real breakthrough in the enzymology of plant secondary metabolism did not occur before the beginning 1970s, particularly supported by two major developments: (i) introduction of column chromatography into protein chemistry with new sorbents, allowing a rapid and selective separation of proteins by size exclusion, ion exchange and affinity chromatography, extending the traditional protein precipitation techniques and (ii) use of plant cell and organ cultures as easy to handle plant *in vitro*-systems. In the early 1970s cell cultures became popular in studies on plant secondary metabolism and the first inducible secondary pathways, e.g. phenylpropanoids, were discovered. At the same time cell cultures of various medicinal plants were established and shown to produce constitutive amounts of secondary metabolites, often at higher levels than the respective mother plant (Zenk, 1991). Although the initial euphoria to utilize plant cells like microorganism as producers of valuable natural products (Barz et al., 1977; Thorpe, 1978) largely vanished after a few years, cell cultures became an excellent and most valuable tool in plant biochemistry.

A number of important biosynthetic pathways were characterized at an enzymatic level in the 1970s and 1980s by the use of cell suspension cultures. Already in 1970, Grisebach and Hahlbrock had begun their successful research on the enzymatic characterization of the biosynthetic pathways of the general phenylpropanoids, as well as the branches leading to flavonoids (Hahlbrock, 1981) and lignins (Grisebach, 1981). Almost all their studies were performed with parsley (*Petroselinum hortense*) cell cultures as major *in vitro* system. Within the heterogeneous group of alkaloids the major classes, for instance, the monoterpenoid indole alkaloids, with more than 2000 structures and the benzylisoquinoline alkaloids, with more than 1000 structures and the tropane alkaloids were characterized at the enzymatic level with great success using tissue or roots cultures from various alkaloid-producing mother plants in the period between the late 1970s and early 1990s (for review see De Luca and Laflamme, 2001; Facchini, 2001; Hashimoto and Yamada, 2003; Zenk and Juenger, this issue).

Despite the highly successful application of cell cultures in biosynthetic research there are classes of secondary products that are not, or less efficiently, produced in dedifferentiated cell cultures. In cases where the biosynthesis is restricted to roots, root organ-cultures have been successfully applied for the enzymatic characterization of the pathways (Robins, 1998). Examples among others are the biosyntheses of nicotine, tropane alkaloids and pyrrolizidine alkaloids. The terpenoids, with more than 30,000 metabolites the most diverse class of plant secondary compounds, need in most cases the differentiated whole plant for an efficient biosynthesis. The characterization of the terpene synthases, the most important enzymes in the formation of mono-, sesqui- and diterpenes, was pioneered in the 1980s by Croteau and Cane who ingeniously linked mechanistic chemistry to enzymology (Croteau, 1987). Two other important pathways that were characterized at the enzymatic level only with whole plants are the cyanogenic glycosides (Møller and Poulton, 1993) and the glucosinolates (Halkier and Gershenzon, 2006).

In the mid 1980s the state-of-the-art in biochemistry of plant secondary metabolism could be summarized as follows: secondary products are *de novo* synthesized from simple precursors of primary metabolism through an elaborated sequence of reactions catalyzed by specific enzymes. There is no evidence that enzymes involved in secondary metabolism include unspecific side-activities of enzymes of primary metabolism or that spontaneous reactions play a role, as sometimes suggested at those days. If a spontaneous reaction occurs (e.g., ring closure) it is under control of the preceding and subsequent enzyme-catalyzed reactions. Like enzymes of primary metabolism, most enzymes involved in plant secondary metabolism show high substrate specificity. This concerns not only the enzymes of core-pathways but also those catalyzing modifying reactions in the course of structural diversification. Examples are the various substrate- and regio-specific *O*-methyltransferases involved in flavonoid metabolism (Ibrahim, 2005). Enzymes of biosynthetic core-pathways frequently comprise multifunctional enzymes like the two cytochrome P450 enzymes involved in the biosynthesis of cyanogenic glucosides, catalyzing the whole sequence of reactions in a channeled manner (Møller and Conn, 1980). Metabolic channeling by organization of biosynthetic enzyme in metabolons (multienzyme complexes) appears to be a common feature of complex biosynthetic pathways of secondary metabolism. Examples are known from phenylpropanoids, terpenoids and alkaloids (Jorgensen et al., 2005). Recently, enzymes which apparently have no equivalent in primary metabolism have become known that are apparently optimized to create chemical diversity. These 'multiproduct enzymes' convert one substrate into an array of products. Examples are certain monoterpene synthases (Fäldt et al., 2003) and sesquiterpene synthases (Steele et al., 1998) from conifers, each generating a few major products accompanied by up to 50 minor ones.

Two other general features of enzymes of secondary metabolism need to be mentioned: (i) they are often found in low concentrations, i.e. specific activities in crude protein extracts are often in the picokat-per-milligram-protein range, whereas those of enzymes of primary metabolism are generally three to five orders of magnitude higher and (ii) they lack feedback regulation as known from biosynthetic pathways of primary metabolism, e.g. amino acids and nucleotides. These features will be considered later.

In the mid 1980s, the first genes encoding enzymes of plant secondary metabolism were cloned and functionally expressed. Again, the phenylpropanoids took the lead: cDNAs for 4-coumarate:CoA ligase, phenylalanine ammonia-lyase and chalcone synthase were successfully isolated and functionally expressed (Kuhn et al., 1984; Ryder et al., 1984; Edwards et al., 1985). The first cDNA of an enzyme of alkaloid metabolism, strictosidine synthase, was sequenced and heterologeously expressed by Kutchan et al. (1988) and the first terpene synthase was cloned and functionally characterized five years later by Colby et al. (1993). The rapidly increasing availability of genes of secondary metabolism nowadays allows the expression of recombinant enzymes and the recovery of protein in substantial quantities for detailed kinetic and structural studies.

The progress in molecular characterization of secondary pathways at the gene level opens new perspectives. In the first place the transfer of genes or whole pathways between organisms could improve and modify medicinal and crop plants or even create plants with new traits. The classical example of metabolic engineering of a plant is the complete transfer of the biosynthetic pathway of a cyanogenic glucoside (dhurrin) from *Sorghum bicolor* into *Arabidopsis thaliana*, making this plant cyanogenic (Tattersall et al., 2001).

Another advantage provided by molecular tools is the cell-specific localization of secondary pathways by immunolocalization of pathway-specific enzymes. Previous results indicate that secondary pathways are not only biochemically well organized but that they are also cell type-specifically expressed – sometimes in cells that are not easily predicted as site for the biosynthesis of secondary compounds. One of the pioneering studies was the localization of hyoscyamine 6-ß-hydroxylase, a specific enzyme in the biosynthesis of tropane alkaloids, in the pericycle of cultured roots of *Hyoscyamus niger* (Hashimoto et al., 1991). Three examples are selected to exemplify the diversity and uniqueness of cell type-specific expression of secondary pathways. The first example is the cellular and subcellular localization of the pathway of monoterpene biosynthesis in the glandular trichomes of peppermint (Fig. 2a). The complete biosynthesis occurs in the secretory cells, from which the essential oil is released into the subcuticular space. In the secretory cell four compartments cooperate in biosynthesis: the plastid harbors the MEP-pathway as well as the enzymes that catalyze the conversion of the active isoprenes to (−)-limonene. Limonene leaves the plastid and the following biosynthetic reactions leading to (−)-menthol as major product are associated with the endoplasmatic reticulum (ER), the mitochondria and the cytosol (Turner et al., 1999; Turner and Croteau, 2004). A second example concerns the localization of homospermidine synthase (HSS), the first pathway-specific enzyme in the biosynthesis of pyrrolizidine alkaloids (Fig. 2b), which in *Senecio* species (Asteraceae, tribe Senecioneae) are synthesized only in the roots. The enzyme is expressed in clusters of endodermis cells and associated cortex cells just adjacent to the phloem, the known path of root-to-shoot transport of the alkaloids (Moll et al., 2002). However, the phylogenetically-related genus *Eupatorium* (Asteraceae, tribe Eupatorieae) differs in its tissue specificity of pyrrolizidine alkaloid biosynthesis. HSS is expressed in all cells of the cortex parenchyma but not in the endodermis (Anke et al., 2004). The third unique example concerns the site of biosynthesis of the benzylisoquinoline alkaloids, including morphine. Although there is still some dispute (Bird et al., 2003; Weid et al., 2004), seven biosynthetic enzymes were localized in the sieve tube elements, while the corresponding gene transcripts were found in the companion cells paired with the sieve tube elements (Samanani et al., 2006). The biosynthetic products like morphine and codeine are stored in multinucleate laticifers that accompany the vascular tissue throughout the plant.

2.2. Functional aspects

Until the 1980s functional aspects of plant secondary metabolism were largely neglected by phytochemists and plant physiologists. In the 1950s, secondary metabolites were regarded as metabolic waste or detoxification products (Peach, 1950; Reznik, 1960). This view changed in the 1970s with the increasing biochemical knowledge of secondary metabolism. Secondary metabolites were now no longer considered as inert end products but as dynamic components of plant metabolism (Barz and Köster, 1980). Typical attributes of plant secondary metabolism were: "expression of shunt and overflow metabolism"; "expression of plants' luxurious metabolism"; "flotsam and jetsam on the metabolic beach"; "playground of biochemical evolution" (Zähner, 1979; Mothes, 1980; Haslam, 1986; Luckner, 1990). An ecological role was generally only accepted due to a coincidental quality of a secondary compound (e.g., toxicity and bitter taste). The only generally accepted

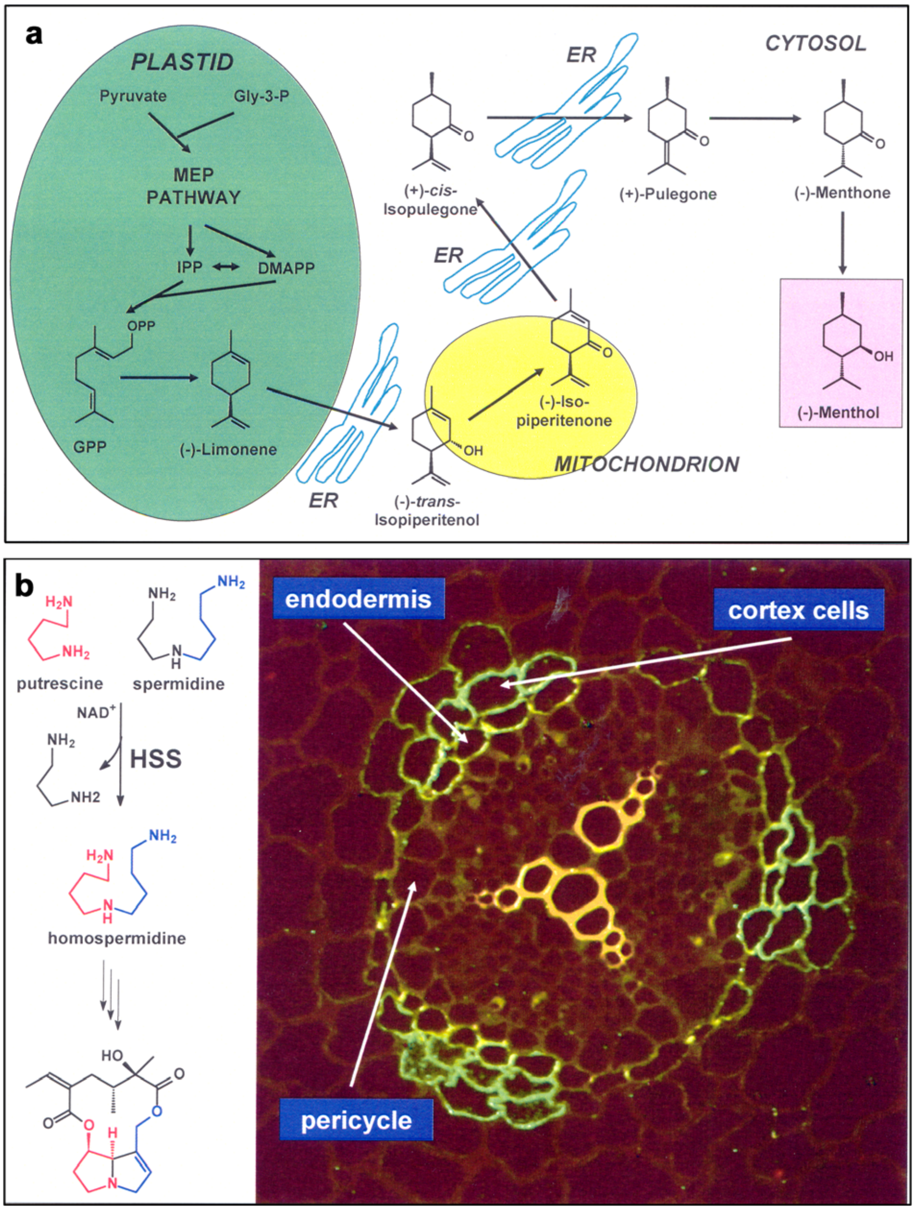

Fig. 2. Examples of cell type-specific expression of pathways of secondary metabolism: (a) biosynthesis of monterpenes in the secretory cells of the glandular trichomes of peppermint. Biosynthesis is associated with four subcellular organelles: plastids (leucoplasts), endoplasmatic reticulum (ER), mitochondria and cytosol (Turner and Croteau, 2004). Courtesy of J. Gershenzon (Jena), modified and (b) homospermidine synthase (HSS) the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis is expressed only in roots. The picture (root cross-section) shows HSS (green immunofluorescence) in defined groups of cells comprising endodermis cells and cortex cells (Moll et al., 2002).

feature of secondary metabolism was its emergence from primary metabolism.

It was not surprising that not phytochemists but entomologists engaged in herbivore-plant interactions changed this view. In his classical paper, Fraenkel (1959) stressed the role of plant secondary metabolites in plant chemical defense and a few years later Ehrlich and Raven (1964) emphasized the importance of reciprocal responses between ecologically closely-linked organisms as a basis of genetic variation in their classical hypothesis on the *coevolution* of butterflies and plants. Their statement:

> ''Indeed, the plant-herbivore 'interface' may be the major zone of interactions responsible for generating terrestrial organic diversity''

highlights the importance of plant secondary metabolism in the everlasting competition between the worlds of autotrophs and heterotrophs on earth. In fact, this idea had already been put forward at the end of the 19th century (Fig. 1). In his paper, rediscovered by Fraenkel (1959), Stahl (1888) described experiments on the chemical protection of plants against slugs and snails and reached the conclusion that plant secondary compounds must have evolved under the selection pressure of herbivores:

> “Hier ist vor allem der Einwendung entgegenzusehen, daß Substanzen wie Gerbstoffe, Bitterstoffe, ätherische Öle, Alkaloide usw. Körper sind, die auch dann vorhanden wären, wenn es gar keine Tiere gäbe. ……… Ihre gegenwärtige quantitative Entwicklung, ihre Verteilung in den Pflanzenorganen, die häufig bevorzugt periphere Lagerung, besonders aber ihr frühzeitiges Erscheinen, können allein aus der Einwirkung der die Pflanzen umgebenden Tierwelt begriffen werden”. (Stahl 1888, p. 566).
>
> “Here the objection has to be countered that compounds like tannins, bitter tasting constituents, essential oils, alkaloids etc. would exist even in a complete absence of animals. ……Their current quantitative status, their distribution within plant organs, their often preferential peripheral accumulation, and particularly, their early appearance can only be explained by the impact of the animal kingdom surrounding the plants”.

Although Stahl’s paper was frequently quoted and even recognized in Pfeffer (1897) famous text book of Plant Physiology he was heavily criticized by many of his colleagues and blamed for his “teleological attitude”. The idea of an ecological function of plant secondary metabolism and its development under the selection pressure of the plants’ environment was almost completely neglected for the following 60 years. Then it became slowly accepted by phytochemists and plant physiologists under the increasing influence of the emerging new discipline of “chemical ecology” (see for instance Harborne, 1972; Rosenthal and Janzen, 1979). Two European pioneers and outspoken advocates of the new ideas were Swain (1977) with his pleading against the “waste products lobby” and Harborne (1972), who organized in 1971 the first Symposium of the Phytochemical Society on “Phytochemical Ecology”.

The functional aspects of plant secondary metabolism are illustrated in Fig. 3. The three cycles were originally published by Reznik (1960) to illustrate the emergence of secondary metabolism from primary metabolism. We just have to add the functional aspect to make the scheme complete (Hartmann, 1996). Secondary metabolism comprises all interactions of plants with their biotic and abiotic environment. The inherent feature of secondary metabolism is its high genetic plasticity and diversity that guarantees flexible adaptations of plants to the demands of their continuously-changing environment. Herbivores or pathogens that during their co-evolutionary adaptations learned to cope with a certain defense barrier may now, as specialists, use a defense compound as a positive signal to recognize the host plant to which they are often socialized. They thus turn a negative chemical signal into a positive one (see for instance Rosenthal and Berenbaum, 1992). Plants may “domesticate” adapted herbivorous insects for pollination and seed dispersal, while a specialist herbivore uses its host plant as food source and for oviposition, or even recruits plant chemical defense compounds, like the pyrrolizidine alkaloids, and utilizes them for its own benefit (Hartmann, 2004).

Following these ideas, it is plausible to realize that plant metabolism exists at two functional levels (Table 1). Primary metabolism covers all processes essential for growth and development whereas secondary metabolism is indispensable for the survival of the individual in its environment.

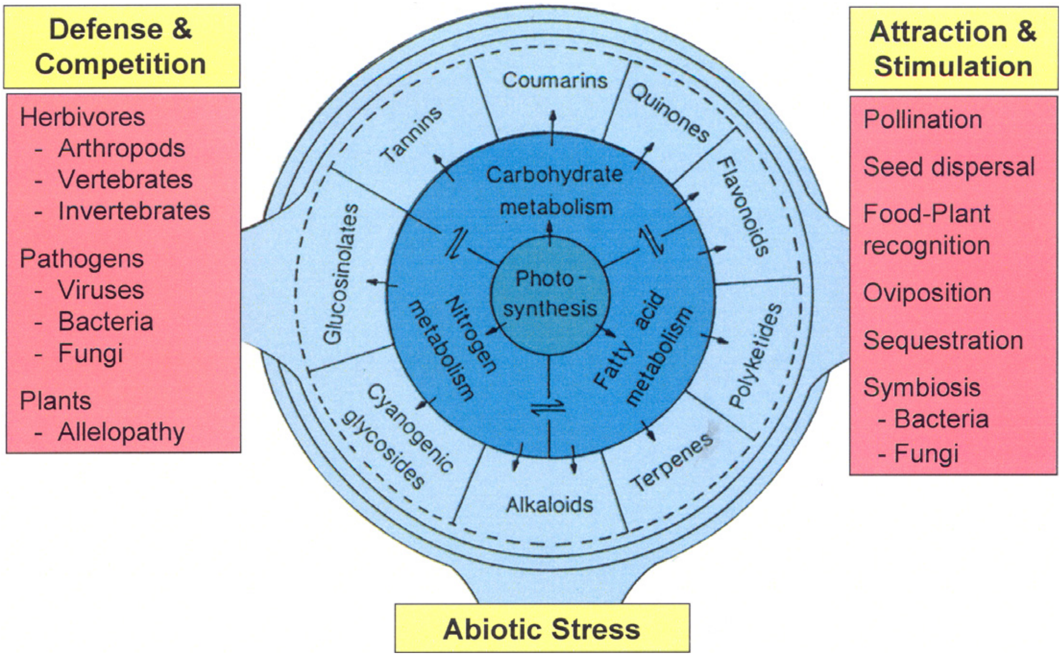

Fig. 3. Ecological functions of plant secondary metabolism.

Table 1
The two levels of plant metabolism

Primary metabolism
Genes with high stringency controlling essential functions
Covers growth and development of the individual
Indispensable
Universal
Uniform
Conservative
Secondary metabolism[a]
Genes with high plasticity controlling functions that are under selection pressure of a continuously changing environment
Covers interactions of the individual with its environment
Dispensable for growth an development
Indispensable for survival in the environment
Unique
Diverse
Adaptive

[a] The term "secondary" is frequently disliked because it may imply "less important". Here, however, the attribute "secondary" addresses a different but by no means less important functional level, analogues to, for instance, the "secondary" structure of a protein that of course is equally important as "primary" or "tertiary" protein structures.

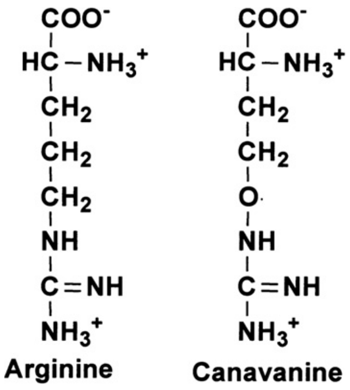

Fig. 4. Canavanine is an arginine antimetabolite found at high levels in seeds of some legumes, e.g. *Canavalia ensiformis*.

Since secondary metabolism is dispensable for growth and development, its components can be continuously modified and adapted to the demands of a continuously changing environmental selection pressure. This high plasticity is mirrored by its attributes (Table 1). The rapidly increasing knowledge about the mechanisms of evolutionary diversification of the genes involved in secondary metabolism is perhaps the most important discovery supporting the ecological function of secondary metabolism. Gene duplication and new functionalization of the duplicate that no longer underlies the stringent functional demands of the mother gene appears to be the key to explain the evolution of metabolic diversity (see Section 2.3.).

During early times of plant evolution, the highly adaptive pool of secondary metabolism apparently also provided a rich reservoir from which compounds may have been recruited that attained essential (primary) functions as phytohormones and signaling compounds within plant metabolism (Table 2). Note that all classes of secondary metabolites listed in Table 2 (terpenoids, phenylpropanoids and flavonoids) already existed before the flowering plants evolved. Recently it was demonstrated that even flavonoids, which are generally regarded as typical secondary compounds with ecochemical functions, may possess primary functions. Flavonoids clearly affect auxin transport and developmental processes in *Arabidopsis* (Buer and Muday, 2004; Taylor and Grotewold, 2005), although it is not yet clear which flavonoids are specifically responsible for these effects.

Table 2
During evolution of the plant kingdom some secondary metabolites have been recruited for primary functions or attained both primary and secondary functions

Compounds of secondary origin that attained primary function	
Diterpenoids	Gibberellines (phytohormone)
Sequiterpenoids	Abscisic acid (phytohormone)
Triterpenoids	Brassinosteroids (phytohormone)
Tertraterpenoids	Carotenoids, xanthophylls (photoprotection)
Flavonoids	Certain flavonoids (developmental regulators)
Benzoates	Salicylate (stress signal)
Compounds which attained both primary and secondary functions	
Lignin	Cell wall strengthening and chemical defense
Canavanine	Chemical defense and seed nitrogen storage

Sometimes a metabolite may include both primary and secondary functions. This is no contradiction and is usually easily explained through the evolutionary background. Two examples are given in Table 2. The dual role of canavanine will be discussed in detail (Rosenthal, 1982, 1991). Canavanine is a toxic arginine-like antimetabolite (Fig. 4) that is accumulated in seeds of certain legumes, e.g. the jack bean (*Canavalia ensiformis*), where it may account for more than 50% of storage nitrogen. Canavanine occurs only in the seeds and its nitrogen is remobilized during germination. Since canavanine efficiently protects the seeds against predators, an ecologist would call it an efficient defense compound, while a plant physiologist would regard it as a nitrogen storage compound. This contradiction is solved by the evolutionary scenario. It is reasonable to assume that the ability to accumulate canavanine evolved under selection pressure of herbivory and that during this process increasing amounts of seed nitrogen were deposited in canavanine. Therefore, coevolution of a mechanism for the remobilization of the canavanine nitrogen during germination was a functional need to prevent shortage in nitrogen supply.

Plants have evolved three major strategies for defense against pathogens and herbivores:

(1) Constitutive accumulation of defense compounds, i.e. metabolites that are stored in the target tissue in concentrations needed to fulfill their functions. Most of the natural products that are isolated in substantial amounts from plants belong to this category.
(2) Constitutive accumulation of preformed defense compounds and 'ignition enzymes' well separated by spatial compartmentation. Upon de-compartmentation,

e.g. by herbivore attack the 'ignition enzyme' liberates the active defense compound. Examples are the liberation of HCN from cyanogenic glycosides; the formation of mustard oils from the glucosinolates; the less-recognized formation of toxic monodesmosidic saponines from non-toxic bidesmosidic saponins stored in plant vacuoles; formation of coumarine from the respective ortho-glucoside; the release of vanilline from vanilloside in *Vanilla* fruits.

(3) Induced formation of defense compounds in response to microbial or herbivore attack. Classical examples are the phytoalexins as plant defense compounds that are specifically produced upon pathogen attack.

Whereas with defense strategies (2) and (3) the causal principle is easily recognized, the efficiency of a constitutive defense can only be judged by the absence or presence of potential attackers. Therefore, evidence supporting a function of a constitutive defense is mostly indirect and often only indicated by the toxicity or deterrence of the compound. Fortunately, during the last decade examples have become known showing that the concentrations of typical constitutive defense compounds could be boosted by supplementary induction caused by herbivores or pathogens (Karban and Baldwin, 1997). This concerns many terpenoids, phenolics, alkaloids (e.g., nicotine), cyanogenic glycosides and glucosinolates. An impressive recently-discovered example is the induced amplification of constitutively expressed oleoresins in conifers upon wounding, e.g. by herbivores such as bark beetles or by pathogens (Keeling and Bohlmann, 2006). This traumatic oleoresin response complex includes *de novo* differentiation of traumatic resin ducts in the developing stem xylem, increased accumulation of monoterpenes, and diterpenes and induced expression of monoterpene and diterpene synthases (Martin et al., 2002). In the conifer this herbivore-induced formation of traumatic resin ducts represents a shift of resource allocation from growth (xylem formation) to defense (resin formation and accumulation).

The basic requirements for constitutive defenses are simple: the defense compound(s) should be stably stored in the target tissues at concentrations needed to prevent damage by antagonists, e.g. pathogen or herbivore. For such a function there is, of course, no need for turnover or degradation of the defense compound. This view is contrary to what was postulated in the 1970s and 1980s when secondary metabolites were no longer regarded as being waste products but were considered as dynamic components of general metabolism (Robinson, 1974; Barz and Köster, 1980) or were suggested to exist 'in a state of dynamic flux within the cell' (Swain, 1977). Indeed, early pulse-chase labeling experiments of terpenoids, phenolics, alkaloids and other secondary compounds appeared to confirm these suggestions and were taken as support for the metabolic dynamics of secondary metabolism, which was thought to exist in continuous degradation and re-synthesis (Robinson, 1974; Coley et al., 1985; Wink and Roberts, 1998). An often quoted example is the rapid turnover of monterpenes synthesized from radioactively-labeled CO_2 observed in peppermint cuttings (Burbott and Loomis, 1969). This experiment was repeated 20 years later and the result with cuttings was confirmed. If intact rooted plants were used, the synthesized terpenoids remained absolutely stable over the time of the experiment (Mihaliak et al., 1991). A thorough and comprehensive evaluation of all published data on turnover of secondary metabolites including terpenoids, phenolic compounds and alkaloids showed that all studies carried out with intact rooted plants lack evidence for turnover while studies with cuttings or isolated plant organs often indicated degradation (Gershenzon, 1994). Obviously, in respect to turnover or degradation, results obtained with isolated plant organs are not reliable, although this phenomenon cannot be satisfyingly explained at the moment. Generally, in almost all experiments loss or disappearance of a tracer compound was already taken as evidence for turnover (Barz and Köster, 1980). Moreover, many studies were performed with cell suspension cultures, which are not adequate systems to study turnover or degradation since the culture medium provides an external lytic compartment with all the hydrolytic enzymes present in cell vacuoles (Wink, 1994).

There are only very few studies in which the assumed degradation pathway has been experimentally characterized. One rare example is the degradation of the antimetabolite canavanine (see above) via a modified urea-cycle in germinating legume seeds (Rosenthal et al., 1989). Considering the extreme diversity of plant secondary metabolism and the fact that each individual system has its own special characteristics, it cannot be excluded that there are some more examples showing turnover particularly of nitrogen-containing compounds. However, turnover and degradation are definitely no common features of secondary metabolism. There is still a lack of substantial physiological data about the dynamics of secondary compounds during plant ontogenesis. To give just one example: The common backbone structure of pyrrolizidine alkaloids in *Senecio* species (Asteraceae) is senecionine *N*-oxide (Fig. 5). Senecionine *N*-oxide is synthesized in the roots (Fig. 2b), distributed all over the plant and structurally diversified, yielding the species-specific alkaloid bouquets. Pulse-chase experiments with radioactively-labeled precursors confirmed that in a growing *Senecio* plant the 'population' of labeled alkaloid molecules remained quantitatively stable over more than four weeks although the alkaloids were spatially mobile and were both reallocated between plant tissues (Hartmann and Dierich, 1998) and diversified by species-specific peripheral reactions (Fig. 5). Pyrrolizidine alkaloids are metabolic end-products. Without awareness of their ecological function their former designation as metabolic waste products is understandable. The earlier-mentioned low specific activities of many enzymes of secondary metabolism and the lack of feedback regulation of secondary pathways is precisely what would be expected

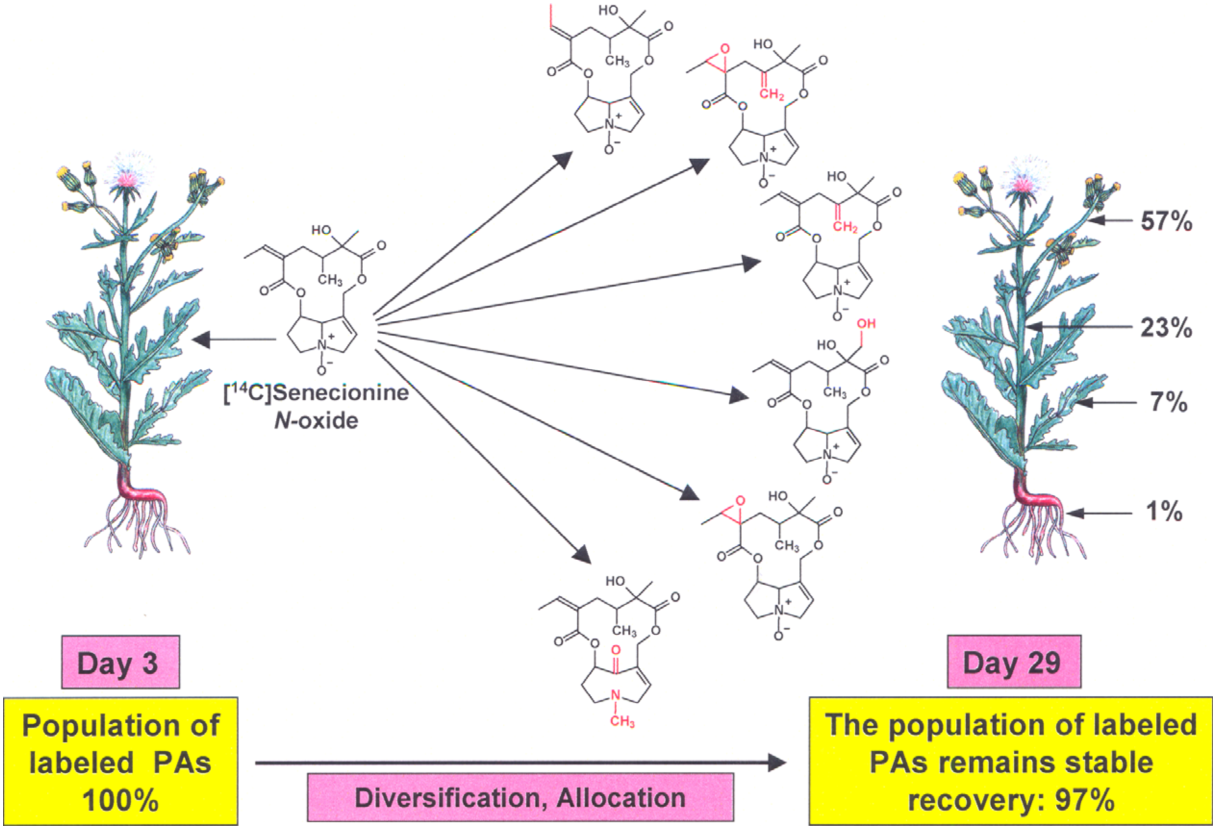

Fig. 5. In some *Senecio* species senecionine *N*-oxide is synthesized as core structure of pyrrolizidine alkaloids. During plant ontogeny it is structurally diversified into the species specific alkaloid pattern and slowly allocated between tissues. As demonstrated in long-term tracer studies, the alkaloids do not show turnover or degradation (Hartmann and Dierich, 1998).

from pathways leading to continuously-accumulating metabolic end-products.

2.3. Evolutionary aspects

In the past about 10 years research, on plant secondary metabolism reached the gene level (Pichersky and Gang, 2000). First, fascinating insights into the creation of genetic diversity of secondary metabolism became visible and then ideas about the mechanisms of gene recruitment and diversification for novel functions arose. Sequence comparisons allowed genes encoding enzymes of secondary pathways to be traced back to their evolutionary origin and their phylogenetic relationships to be established. After more than 100 years of ignorance, biologists finally recognized the causal connection between gene diversity and plasticity of secondary metabolism in its indispensable ecological role in the dynamic interactions of the plant kingdom with its continuously changing environment.

Gene duplication is assumed to be the major driving force for gene diversification and recruitment (Pichersky and Gang, 2000; Ober, 2005). If a gene that directs an essential function is duplicated, the duplicate is released from the stringent function of the mother gene; it may either be eliminated by inactivation (e.g., pseudogenization) or recruited for modified or new functions. There are at least two general possibilities how duplicate genes are recruited and functionalized: (i) continuous modification of its function during plant speciation, as consequence of which large gene families with rich functional diversity arise and (ii) the duplicate is recruited for a new stringent function in a novel biochemical environment, as consequence, a new single copy gene originates.

Plant polyketide synthases (PKSs) (Austin and Noel, 2003) should exemplify the first possibility. Fig. 6 shows a phylogenetic tree of angiosperm type III PKSs (Liu et al., 2007). The two clusters repesent chalcone synthases (CHSs) (Fig. 6B) and non-chalcone-synthases (Fig. 6A) of angiosperms. The latter apparently originated from ancient duplication of an ancestral CHS gene. During angiosperm speciation one gene retained the essential CHS function while the duplicate underwent functional diversification. (Fig. 6A and C). Under environmental selection pressure new polyketide synthases evolved, producing a rich diversity of polyketides that can be distinguished by their biogenetic starter units (marked red in Fig. 6C), the number of added C$_2$-units and the mechanism involved in cyclization. Even more complex and diverse gene families represent hundreds of terpene synthases (TPSs) (Tholl, 2006). The TPSs from angiosperms and gymnosperms form distinct families that appear to have arisen from an ancestral diterpene synthase involved in

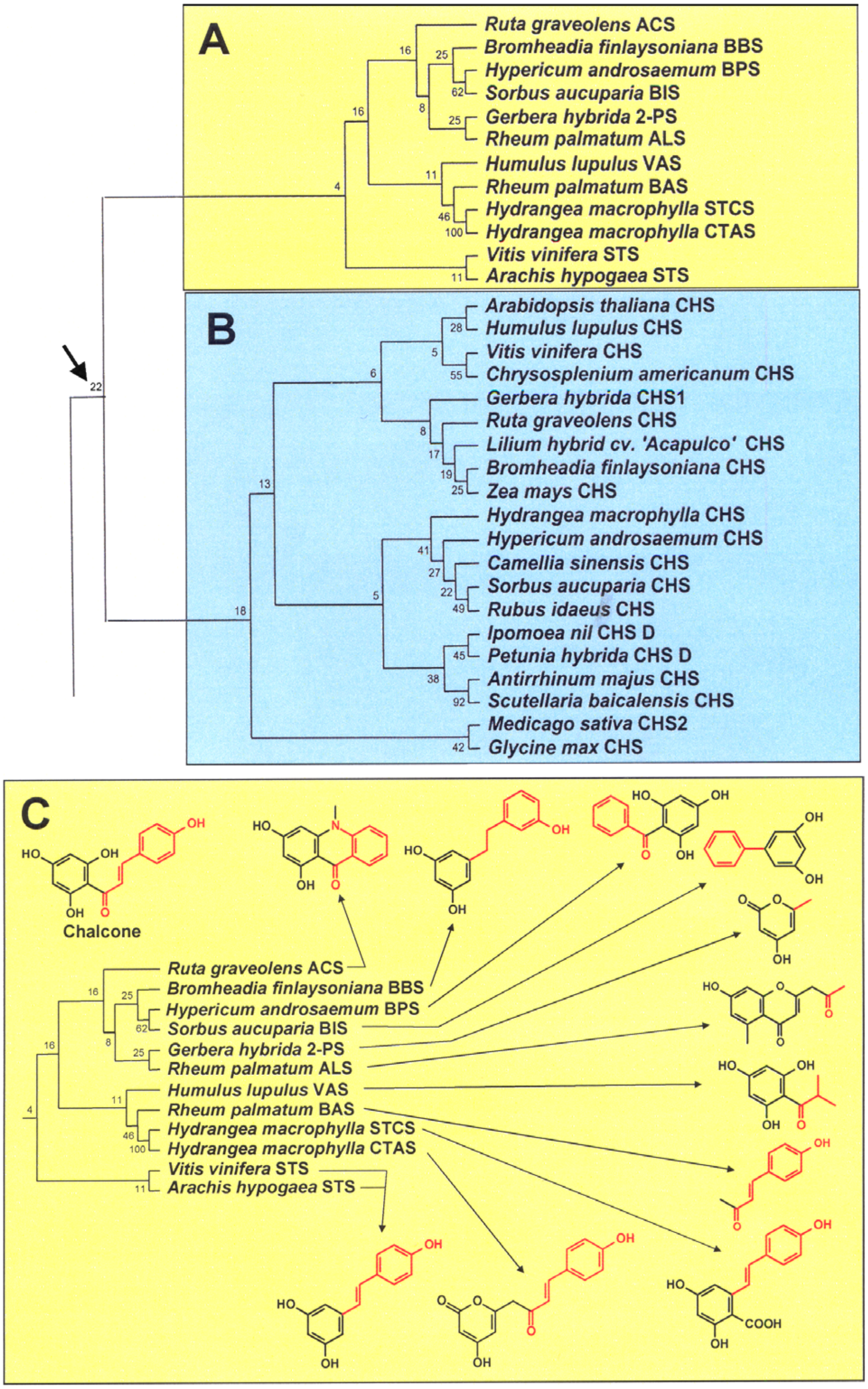

Fig. 6. Neighbour-joining trees of angiosperm type III polyketide synthases (PKSs). Before speciation of angiosperms the gene encoding chalcone synthases (CHS) clade B (shaded blue) duplicated. The gene duplicate was functionally diversified (yellow shaded clade of non-chalcone-synthases) during angiosperm speciation. Functional diversification (C) concerns starter units (red marked) and modification of cyclization modes. ACS, acridone synthase; BBS, bibenzyl synthase; BPS, benzophenone synthase; BIS, biphenyl synthase; 2-PS, 2-pyrone synthase; ALS, aloesone synthase; VAS, valerophenone synthase; BAS, benzalacetone synthase; STCS, stilbene carboxylate synthase; CTAS, 4-coumaroyltriacetate lactone synthase; STS, stilbene synthase (Liu et al., 2007). Courtesy of L. Beerhues (Braunschweig), modified.

primary metabolism (Trapp and Croteau, 2001; Martin et al., 2004). Various plants have been shown to possess large TPS gene families (e.g., 30 TPS in *A. thaliana*) resulting from cycles of gene duplication, multiple mutations and functional divergence. Apparently these multigene families and their differential expression are mediated by

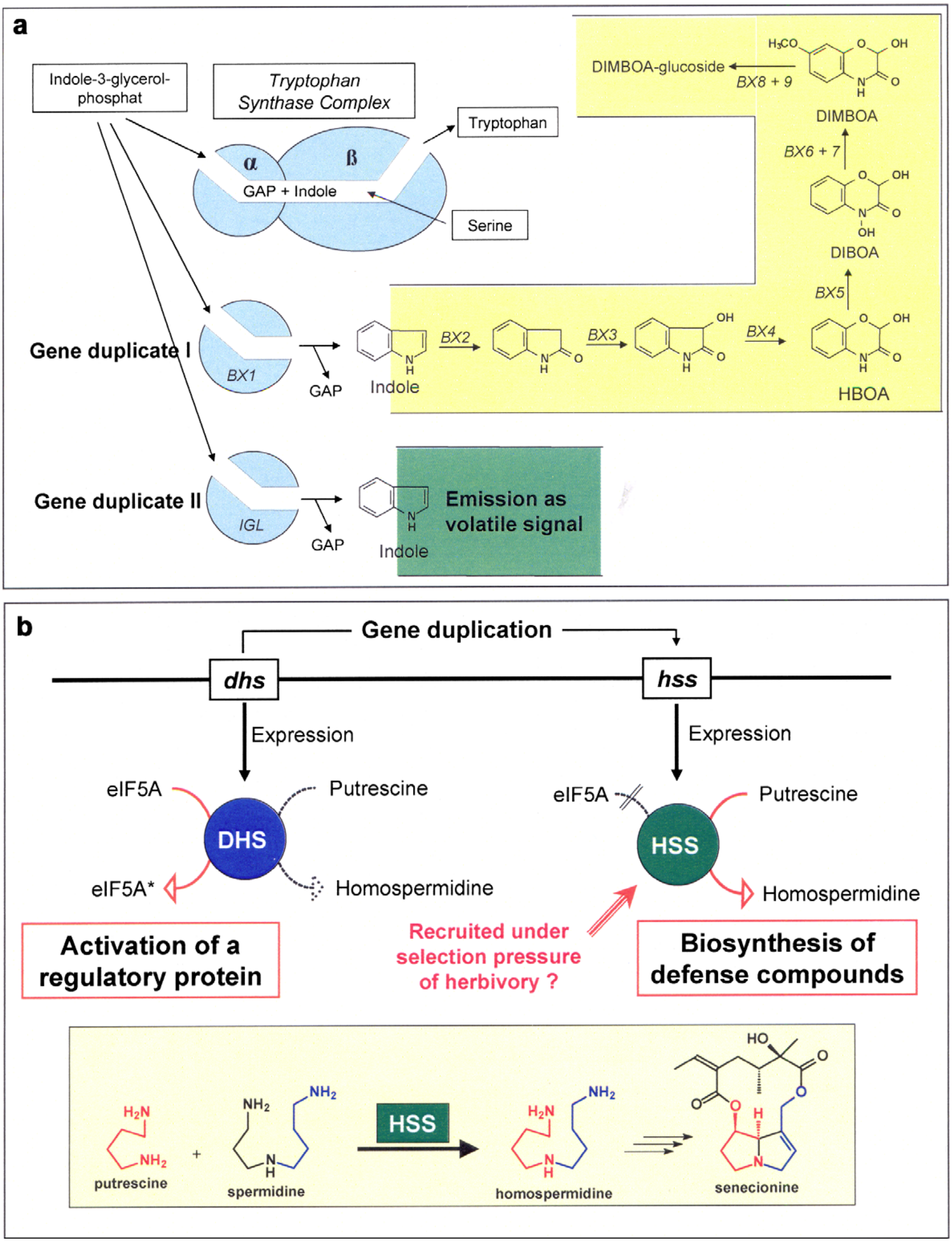

Fig. 7. Recruitment of genes of primary metabolism for new functions in secondary metabolism. (a) Example benzoaxazinone (DIMBOA) and indole biosyntheses in maize. The gene encoding the α-subunit of tryptophan synthase duplicated twice. One duplicate was integrated (BX1) into DIMBOA biosynthesis the other into indole formation (IGL, indole-3-glycerol phosphate lyase). DIMBO is synthesized as defense compound under developmental control in young seedlings. Formation of indole as a signal of indirect defense is later induced in leaves in response to herbivore damage (Gierl and Frey (2001). (b) Example homospermidine synthase HSS in *Senecio*. (HSS) is derived by gene duplication from deoxyhypusine synthase (DHS), an enzyme involved in the activation of a regulatory protein (eIF5A). HSS retained the side activity of DHS to produce homospermidine and was integrated into the biosynthetic pathway of plant defense compounds, i.e. pyrrolizidine alkaloids (senecionine) (Ober et al. 2002).

developmental and stress-related programs, creating the known complexity and variability of terpene formation in plants (Tholl, 2006). Many other fascinating examples of functional diversification of genes are becoming visible including glucosyltransferases of secondary metabolism (Strack et al., 2003; Gachon et al., 2005), *O*-methyltransferases of flavonoid metabolism (Ibrahim, 2005) and the enzymes catalyzing triterpene cyclization (Phillips et al., 2006).

The second possibility of how gene duplicates can acquire novel functions is less frequently known. The evolution of benzoxazinone and indole provide an impressive example (Gierl and Frey, 2001). The biosynthesis of DIMBOA (Fig. 7a) has been completely characterized on the molecular level. It is one of the rare cases in eukaryotes where non-homologous genes are organized in a gene cluster. The first gene in the sequence (*BX1*) originated from a duplication of the gene encoding the α-subunit of ubiquitous tryptophan synthase. A second gene duplicate was recruited for the emission of volatile indole. Both gene duplicates acquired completely new functions. The first within the biosynthesis of defense compounds under developmental control of young seedlings. The second, encoding indole-3-glycerol phosphate lyase (IGL), is specifically induced in mature leaves in response to herbivore damage. IGL catalyzes the formation of indole as one of the volatiles in maize emitted as signals in tritrophic defense: the volatile signals allure parasitoids to their prey, i.e. the maize herbivores (Frey et al., 2000). A second example concerns homospermidine synthase (HSS), which has already been mentioned as the first pathway-specific enzyme in the biosynthesis of pyrrolizidine alkaloids (Fig. 2b). HSS evolved by duplication of the gene encoding desoxyhypusine synthase (DHS) (Ober and Hartmann, 1999). DHS takes part in the posttranslational activation of the eukaryotic initiation factor 5A (eIF5A). HSS retained all kinetic and molecular properties of DHS except the ability to bind the eIF5A precursor protein (Ober et al., 2002). The ability of synthesizing homospermidine from putrescine and spermidine, a side-activity already existent in DHS, became the core-activity of HSS (Fig. 7b). Again the duplicate of an essential gene of primary metabolism was recruited for an entirely different function in secondary metabolism. A third example concerns acyltransferases that operate with 1-*O*-ß-acetalesters (1-*O*-glucose esters) as acyl donors instead of coenzyme A thioesters. These enzymes, which play an important role in plant phenylpropanoid metabolism, are most likely evolved from serine carboxypeptidases by gene duplication and new functionalization (Milkowski and Strack, 2004; Stehle et al., 2006).

3. Perspectives

Within 50 years a bewildering array of waste products turned into a classified selection of chemical structures with indispensable ecological functions. The immense chemical diversity of plant secondary products – formerly understood as evolutionary play or matter of chance – is now recognized as an essential part of the strategies of plants to cope with the adversities of a hostile environment. Each plant population possesses its unique set of secondary compounds well adapted to the particular demands of the plant's ecological niche. The chemical diversity of secondary metabolism simply reflects the diversity of the organisms with all their morphological particularities, which we are well aware from plants, animals and microbes.

From the mechanistic point of view, the increasing knowledge of molecular gene information and the great impact of the genomic tools now available will advance the complete molecular characterization of secondary pathways as a base for their biotechnological application in medicinal and agricultural directions. The knowledge that secondary metabolites are functionally optimized under the ecological demands of plants (e.g., protection against animals and pathogens) should provide a valuable background. The emerging tools of transcript and metabolic profiling will be important to learn more about the dynamic integration of often extremely variable secondary pathways into the plants' metabolic network. First ideas of how this metabolic diversity is regulated in a coordinate manner by transcriptional control are just emerging (Broun, 2005). Most likely duplication and functional divergence of transcription factors will open a new level affecting the rapid evolution of new or modified metabolic pathways from existing ones (Grotewold, 2005).

From the functional point of view, the powerful molecular tools now available provide a substantial base from which to explore the role of secondary metabolism in the diversity of plant–environment interactions. We are still only beginning to understand the whole complexity of, for instance, plant–herbivore interactions. A pioneering attempt is the fusion of approaches of ecology and molecular biology by Baldwin and his colleagues (2001), who are studying gene expression in natural populations of plants (e.g., *Nicotiana attenuata*). Microarray gene expression profiling will be a powerful tool to recognize changes of gene activity under environmental stress as, for instance, demonstrated for conifer defense upon attack of herbivorous insects (Ralph et al., 2006): among others the activities of genes of octadecanoid and ethylene signalling pathways, terpenoid biosynthesis, and phenolic metabolism were upregulated.

The recent exciting first insights into the mechanisms of gene recruitment, diversification and new functionalization has brought secondary metabolism back to the forefront of biological research of biology. To trace back genes of secondary metabolism to their origin, identify their ancestors and, perhaps, reconstruct the evolution of whole secondary pathways will be a challenging but realistic future goal. We will learn more about the molecular mechanisms and strategies mediating the dynamic adaptations of plants under the selection pressure of a continuously changing environment. Such insights into the genetic manifestation of evolutionary events will go far beyond the specific relevance of secondary pathways and their products. This new approach requires not only a proper handling of the sophisticated molecular tools, but also a precise knowledge of the individual life histories of the organisms involved.

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