Chapter 10
Secondary Metabolism

10.1 Introduction

The phenomenon of secondary metabolism was already recognized in the early phases of modern experimental botany. In his textbook published in 1873, Julius Sachs, one of the great pioneers 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 innere Ökonomie der Pflanze ist bis jetzt nicht bekannt" (Sachs 1873, p. 641). Translation: "We can designate as by-products of metabolism such compounds that are formed by metabolism, but that are no longer used for the formation of new cells. Any importance of these compounds for the inner economy of the plant is as yet unknown". This clear statement is still valid. Sachs did not refer to any functions of the by-products, today known as secondary products (see review by Hartmann 1996).

Plants form an important part of our everyday diet, and their constituents have been intensively studied for decades. In addition to essential primary metabolites (e.g., carbohydrates, lipids, and amino acids), higher plants are able to synthesize a wide variety of low molecular weight compounds—the secondary metabolites (Fig. 10.1). The production of these compounds is often low (less than 1% of dry weight), and depends strongly on the physiological and developmental stage of the plant.

Although plant secondary metabolites seem to have no recognized role in the maintenance of fundamental life processes of the plants that synthesize these, they do have an important role in the interaction of the plant with its environment.

To study secondary metabolism per se is an exciting area of plant physiology, or actually of botany in general. Moreover, many of its constituents are important substances of medical interest and other areas of human life, and therefore in vitro studies on this topic were soon of commercial interest. Investigations focused on metabolites to be produced by cultured cells of some plant species producing commercially highly valuable chemicals (Zárate and Yeoman 2001). These investigations included, e.g., the characterization of several hundred enzymes also as a contribution to basic interests. Still, due to the economic importance of this topic, in the following commercial aspects will dominate.

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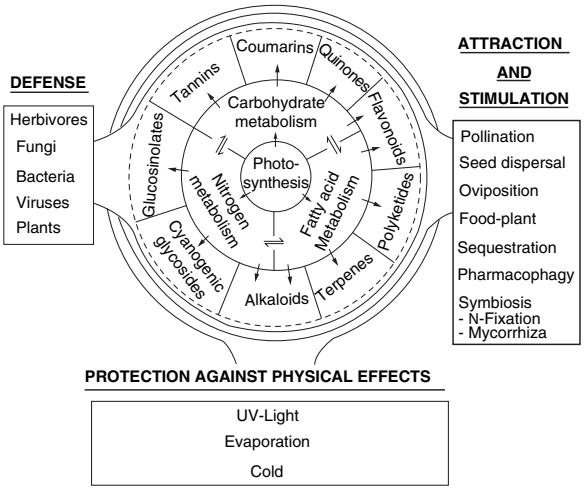


Fig. 10.1 Secondary metabolites originate from common precursors as products of primary metabolism. Three basic metabolic processes governed by photosynthesis, i.e., nitrogen metabolism, fatty acid metabolism, and carbohydrate metabolism, are responsible for the synthesis of secondary metabolites like alkaloids, terpenes, polyketides, flavonoids, quinones, coumarins, tannins, glucosinolates, and cyanogenic glycosides. Their functions involve all aspects of a plant's chemical interactions with the environment (from Hartmann 1996)

At least one fourth of all prescribed pharmaceuticals in industrialized countries contain compounds that are directly or indirectly, via semi-synthesis, derived from plants. Many of these pharmaceuticals are still in use today, and often no useful synthetic substitutes have been found that possess the same efficacy and pharmacological specificity. Furthermore, 11% of the 252 basic and essential drugs considered by WHO are exclusively derived from flowering plants (Rates 2001). Misawa (1991) reviewed the production of secondary metabolites in plant tissue culture in an FAO bulletin. Indeed, prescription drugs containing phytochemicals were valued at more than US$ 30 billion in 2002 in the USA (Raskin et al. 2002).

Based on their biosynthetic origins, plant secondary metabolites can be structurally subdivided into five major groups (Fig. 10.2): polyketides, isoprenoids (e.g., terpenoids), alkaloids, phenylpropanoids, and flavonoids.

1. The polyketides are produced via the acetate–mevalonate pathway;
2. the isoprenoids (terpenoids and steroids) are derived from the five-carbon precursor isopentenyl diphosphate (IPP), produced via the classical mevalonate pathway, or the novel MEP (non-mevalonate or Rohmer) pathway;
3. the alkaloids are synthesized from various amino acids;
4. phenylpropanoids having a C6–C3 unit are derived from aromatic amino acids, phenylalanine, or tyrosine;
5. flavonoids are synthesized by the combination of phenylpropanoids and polyketides (Verpoorte 2000).

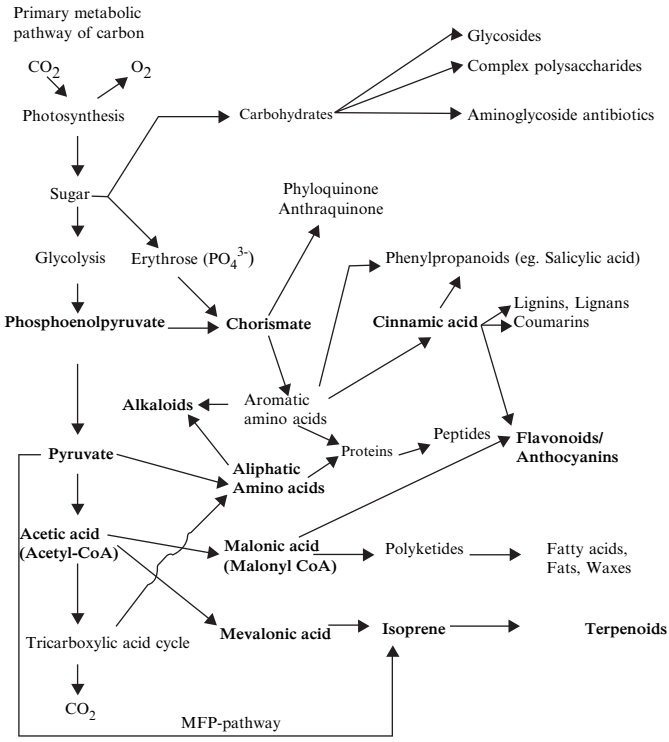


Fig. 10.2 Major pathways of biosynthesis of secondary metabolites, viz. polyketides, isoprenoids (e.g., terpenoids), alkaloids, phenylpropanoids, and flavonoids (after Verpoorte 2000)

10.2 Mechanism of Production of Secondary Metabolites

There are some basic metabolic pathways for the synthesis of secondary metabolites, as shown in Fig. 10.3. These metabolites form five major groups, as mentioned above.

One possible way to classify the 12,000 known alkaloids is to further subdivide these into the following 15 subclasses: proto-, piperidine, pyrrolidine, pyridine, quinolizidine, tropane, pyrrolizidine, imidazole, purine, quinoline, isoquinoline, quinazoline, indole, terpenoid, and steroidal alkaloids.

Secondary metabolism is an integral part of the developmental program of plants, and the accumulation of secondary metabolites can demarcate the onset of developmental stages. However, only a few pathways (e.g., flavonoids, and terpenoid indole and isoquinoline alkaloids) in plants are well understood today, after many years of classical biochemical research (e.g., Street 1977; Staba 1980; Dixon and Steele 1999; Hashimoto and Yamada 2003; Vanisree and Tsay 2004; Vancanneyt et al. 2004; Vanisree et al. 2004).

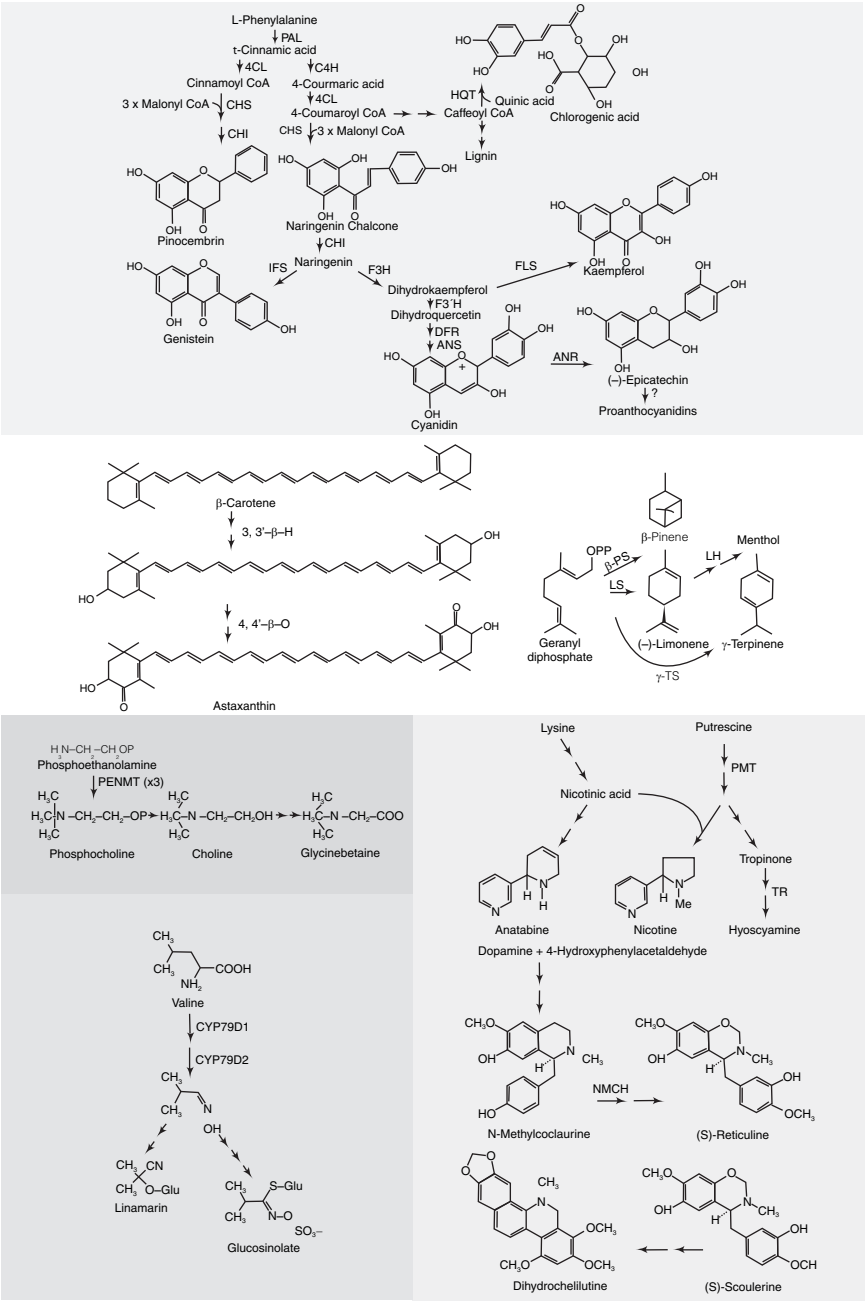


Fig. 10.3 Schematics of pathways for the production of natural products

Detailed biosynthetic pathways of these metabolites are beyond the scope of this book. Thus, a brief outline of various key compounds within plants, and of their biosynthetic pathways will be given.

Secondary metabolites belonging to a given subclass are not always synthesized from the same primary metabolites, but their chemical structures share the same basic skeleton. Cinnamic acid and its simple derivatives are the common precursors of key intermediates of the various phenylpropanoid classes illustrated. In turn, the class-specific key intermediates are structurally diversified to yield 1,000s of individual compounds (Hartmann 1996).

Because of the activity of enzymes with different substrate- and stereo-specificity, the chemical diversity and biological activity of the molecules belonging to a given subclass can be enormous (Tulp and Bohlin 2002). For example, various types of cyclic monoterpenes are synthesized from the common precursor geranyl diphosphate by action of specific monoterpene cyclases. Some subclasses are found only in a few plant families (e.g., medicinal tropane alkaloids are found only in the Solanaceae and Erythroxylaceae), whereas flavonoids, for example, are widely distributed throughout the plant kingdom. The concept of combinatorial biochemistry is based on the fact that different plants, either closely or more distantly related, synthesize structurally similar, but nevertheless diverse molecules. As such, it can be expected that an enzyme with a certain substrate specificity isolated from one plant might encounter new, but related substrates when introduced into another plant. This has been experimentally proved, as given below (Sato et al. 2001). Thus, by introducing genes involved in the biosynthesis of a given compound isolated from one plant into another plant synthesizing related molecules, new chemical structures not previously found in nature may be obtained.

Successful attempts of insertion of more than one gene of a known pathway into a host organism have also been reported. For instance, following particle bombardment of tobacco leaves and plant regeneration, the expression of two consecutive genes involved in the terpenoid indole alkaloid pathway of *Catharanthus roseus* has been reported; *C. roseus* is a well-known species able to accumulate the two potent anticancer drugs vincristine and vinblastine encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR1) in tobacco plants (Leech et al. 1998). TDC and STR1 are two adjacent pathway enzymes that together form strictosidine, which is an important intermediate of over 3,000 indole alkaloids (Fig. 10.4), many of which possess important pharmaceutical properties. Both tdc and str1 genes are absent in tobacco plants. Analysis of transgenic plants at the RNA and DNA levels demonstrated a range of integration events and steady-state transcript levels for both transgenes, beside a 100% co-integration of both transgenes (Zárate and Yeoman 2001). Similarly, a gene involved in the terpenoid indole alkaloid pathway of *C. roseus*, sgd (cf. strictosidine β-D-glucosidase; Fig. 10.4), has been introduced via *Agrobacterium tumefaciens* and expressed in suspended tobacco cells (Zárate 1999).

There is also an account where a whole heterologous secondary metabolic pathway was expressed in a host plant (Ye et al. 2000) following *A. tumefaciens*-mediated transformation. Ye et al. (2000) introduced the entire β-carotene biosynthetic pathway,

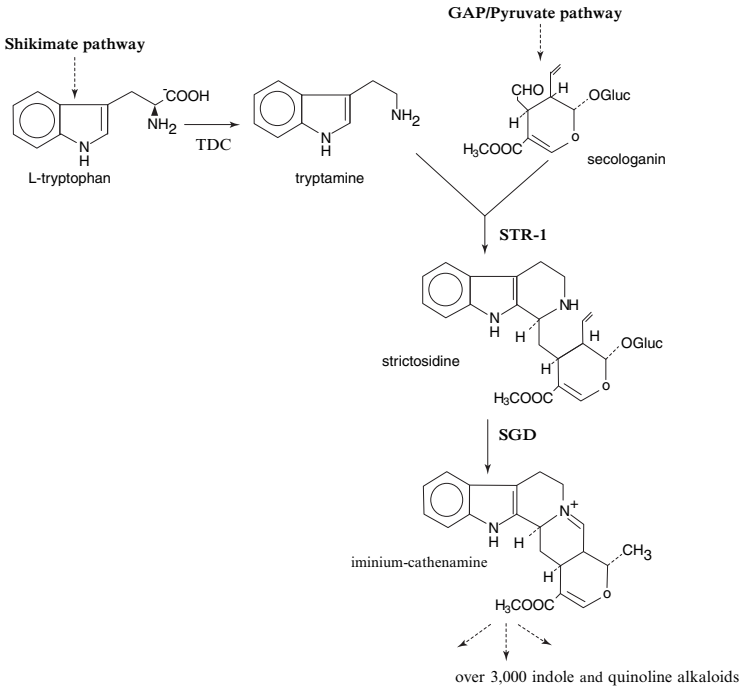


Fig. 10.4 Partial illustration of the biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus* leading to the formation of the intermediate strictosidine, central precursor of over 3,000 indole and quinoline alkaloids. *TDC* Tryptophan ecarboxylase, *STR-1* strictosidine synthase, *SGD* strictosidine lucosidase, *GAP* glyceraldehyde-3-phosphate (Zárate and Yeoman 2001)

vitamin A precursor, into rice endosperm in a single transformation effort with three vectors harboring four transgenes: psy, plant phytoene synthase, crt-1, bacterial phytoene desaturase, lcy, lycopene β-cyclase, and tp, transient peptide. In most cases, the transformed endosperms were yellow, indicating carotenoid formation, and in some lines β-carotene was the only carotenoid detected. This elegant report illustrates how the nutritional value of a major staple food may be augmented by recombinant DNA technology.

For further details on genetic transformation, the reader is referred to Section 13.2.

10.3 Historical Background

In contrast to primary metabolism of cell cultures where only limited investigations have been carried out, the literature is full of investigations on secondary metabolism. This difference in the variability of information is due to the fact that the intermediate products and end products of primary metabolism can be obtained from agriculture in huge amounts at low costs, in contrast to secondary plant products of high value that fetch high prices for even small amounts to be used

in cosmetic or pharmaceutical industries (Charlwood et al. 1990; Misawa 1991; Komamine et al. 1991; Neumann 1995; Bender and Kumar 2001; Alfermann et al. 2003; Vanisree and Tsay 2004; Vanisree et al. 2004; see also Kumar and Roy 2006, Kumar and Sopory 2008, and Kumar and Shekhawat 2009). Bourgaud et al. (2001) reviewed the historical perspective of plant secondary metabolite production.

To date, there has been continuous increase of patents filed for products based on tissue culture by commercial companies. These include additives to food, and pigments. These substances were often obtained from raw materials imported from tropical and subtropical regions. To ensure continuous production, storage of significant amounts of these raw materials is required, associated with considerable costs and risks. In addition, they can vary strongly in quality, depending on the year of production and the regions of export, and also in price, depending on economic considerations like changes in world market prices. All these factors have stimulated the production of secondary products under controlled conditions in plant tissue culture laboratories near the commercial unit, to produce the final product for the market.

By the beginning of the 1970s, plant cell culture had attained a developmental status employing methods of microbial fermentation techniques—e.g., antibiotic production to be used for large-scale cultures from plants, in order to avoid the above mentioned problems of imports of raw materials. Today, up to 30% of medical prescriptions are based on plants, or contain plant components. Traditional medicinal systems utilize plant-based medicines, and are experiencing a revival worldwide. This has resulted in enormous pressures on biodiversity, and the destruction of valuable biotopes particularly in developing countries involved in meeting the demands of global markets. Tissue culture could provide alternatives.

Among the plant-derived compounds are two drugs derived from the Madagascar periwinkle (*Catharanthus roseus*): vinblastine and vincristine. Other examples of important drugs derived directly, or indirectly from plants include the anticancer drugs paclitaxel (Taxol), podophyllotoxin, and campthothecin, the analgesic drug morphine, and semi-synthetic drugs such as the vast group of steroidal hormones derived from diosgenin. There is revival of interest in plant secondary metabolites, as there has been only limited success of combinatorial chemistry or computational drug design to deliver novel pharmaceutically active compounds (Müller-Kuhrt 2003).

The products of highest market interest are based on glycosides and alkaloids. Beside these, steroids, enzymes, and pigments are of considerable interest. Table 10.1 provides some of the important plants and their products that have a potential for use in tissue culture.

Only few plant materials were used at the beginning, i.e., systems for the production of heart alkaloids from *Digitalis*, and atropine and scopolamine from *Datura* cultures. *Lithospermum* produces antimicrobial agents, shikonin being of particular importance (Yamamura et al. 2003). The synthesis of methyldigoxin by hydroxylation of methyldigitoxin was another goal (Alfermann et al. 1985). *Coptis* is used for making tonics of berberines.

Due to the increased appeal of natural products for medicinal purposes, metabolic engineering can have a significant impact on the production of pharmaceuticals, and help in the design of new therapies. The candidate plant cell cultures are

Table 10.1 Compounds of industrial interest produced in plant tissue culture

S. no.	Effects	Plants
1	Antimicrobial effects (virus) (protozoan) (bacteria) (bacteria)	*Agrostemma*/*Phytolacca* *Catharanthus* *Lithospermum* *Ruta*
2	Antitumor effects	*Camptotheca*, *Antharanthus*, *Maytenus*, *Podophyllum*, *Taxus*, *Tripterygium*
3	Painkillers	*Chamomilla*, *Valeriana*, *Papaver*
4	Enzymes for proteolysis	*Papaya*, *Scopolia*, *Ananas*
5	Enzymes for biotransformation	*Cannabis*, *Digitalis*, *Lupinus*, *Mentha*, *Papaver*
6	Appetizers or taste enhancers	*Asparagus*, *Apium graveolens*, *Allium*, *Capsicum*, *Sinapis*
7	Hydrocarbon-yielding	*Asclepias*, *Euphorbia*
8	Sweeteners	*Glycorrhiza*, *Hydrangea*, *Stevia*
9	Tonics	*Bluperrum*, *Cinchona*, *Coptis*, *Phellodendron*, *Panax*
10	Insecticides	*Derris*, *Pyrethrum*

generally chosen by screening from medicinal and aromatic plants already used in drug production. At present, research and development are focused on plants producing substances with immunomodulating, antiviral, antimicrobial, antiparasite, antitumor, anti-inflammatory, hypoglycemic, tranquilizer, and antifeedant activity (Yamada 1991).

The last 15 years have produced a large quantity of results on the biosynthetic pathways leading to secondary metabolites. Concomitantly, at the beginning of the 1990s, a new discipline called metabolic engineering appeared. According to Bailey (1991), metabolic engineering is "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology". In many cases, this approach relies on the identification of limiting enzyme activities after successful pathway elucidation and metabolite mapping (metabolomics). Such limiting steps are improved with an appropriate use of genetic transformation. Most of the strategies developed so far are based on the introduction of genes isolated from more efficient organisms, promoters that enhance the expression of a target gene, or antisense and co-suppression techniques for the obtainment of plants with the desired traits. In addition to their synthesis as such, the transport of metabolites within the plant system, and its localization play a key role in optimizing the yield. Recently, attempts have been made to understand the regulation of transport (Yazaki 2005).

Quite some time ago, Yeoman et al. (1980) suggested an interesting model to influence the synthesis of secondary products (Fig. 10.5). In this model, W is the immediate precursor of the substance X to be produced, and P an unspecific precursor from which X can be derived following the production of Q, the first specific intermediate in the pathway eventually producing X.

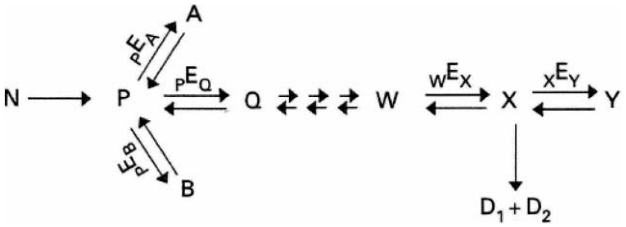


Fig. 10.5 Model to regulate secondary metabolism (Yeoman and Yeoman 1996)

Based on this model, there are several possibilities to promote the synthesis of X as the desired product. For a start, optimizing the metabolic intensity of the cultures will establish the basic production of X. Moreover, P can be diverted to alternative pathways symbolized as A and B. The entrance of P into the metabolic pathway specific for the synthesis of X can be limited by a low activity of the enzymes pEQ, or the following enzymes. Finally, also X could simply be an intermediate of the synthesis of Y. Consequently, its concentration would be determined by the activity of the two enzymes WEX and XEY as an equilibrium of the synthesis of X and Y, and at a given time a given concentration of X would be determined. The concentration of X will also be influenced by direct breakdown (D1+D2), or by fixation as a conjugate (K with other molecules). Especially the formation of conjugates has been investigated quite extensively these recent years.

Based on this (certainly too) simple model, some conclusions can already be drawn to initiate more detailed investigations. One possibility to promote the reaction chain P–Q–W–X is the application of Q to the nutrient medium, this being the first pathway-specific intermediate. In terms of simple enzyme kinetics, it can be assumed that the reaction P to Q will be inhibited by an excess of P in the cells. Another possibility to promote the pathway to produce X is a supplement of A, B, or Y. Making use of various possibilities to influence the production of the target substance requires knowledge of the metabolism of this compound, as well as of the pool size of the various molecules, and the equilibrium conditions of the enzymes involved. As described, such information is available for some cell culture systems (see also below). The Yeoman group used this model as a basis to optimize capsaicin production. Into this scheme, it would be of interest to include changes in enzyme availability following gene technological manipulations of the cells.

Basically, the assumptions of the model have been confirmed also in our own studies to produce atropine and scopolamine in *Datura* cultures. These are the two main alkaloides of this species, synthesized from the two amino acids phenylalanine and ornithine, and symbolized as P in the model. The latter are transformed via tropine and tropic acid into atropine, and finally into scopolamine. Tropine and tropic acid are symbolized as Q/W in the model. At a supplement of ornithine or phenylalanine, or both, to the *Datura* cultures (symbolized as P), only the

Table 10.2 Influences of some precursors of the synthesis of tropane alkaloids on alkaloid concentration (μg/g dry wt.) of haploid cell suspensions of *Datura innoxia* Mill. (application of precursors for 1 week after 3 weeks pre-culture)

	Tropine	Atropine
Control	15	0
+Leucine and glycine	10	0
+Ornithine and phenylalanine	90	Traces
+Tropine and tropic acid	175	75

concentration of tropine is increased, i.e., of Q/W. An application of tropic acid and tropine, however, results in an increase in the atropine concentration (Table 10.2), in other experiments also of scopolamine (Forche, unpublished results of our institutes; see Neumann 1995).

Several other laboratories have reported secondary metabolite production from plant tissue cultures (Carew and Staba 1965; Khanna and Staba 1968; Khanna 1977; Barz et al. 1977; Kiebler and Neumann 1980; Neumann et al. 1985; Alfermann and Reinhard 1986; Furuya 1988; P.R. Holden et al. 1988; Holden 1990; Vasil 1991; Abe et al. 1993; Neumann 1995; Datta and Srivastava 1997; Jain et al. 1998; Jacob and Malpathak 2006; Narula et al. 2006; Hiroaka and Bhatt 2008; Kukreja and Garg 2008; Sonderquist and Lee 2008; Jacob et al. 2008; Sharada et al. 2008; Srivastava et al. 2008). Vanisree et al. (2004) and Dixon (2005) reviewed the production of secondary metabolites in tissue culture and engineering of natural product pathways, respectively.

More than 50 years ago, Routien and Nickel (1956) suggested the potential for the production of secondary metabolites in culture, and received the first patent. Later, the National Aeronautics and Space Administration (NASA) started to support research on plant cell cultures for regenerative life-support systems (Krikorian and Levine 1991; Krikorian 2001). Indeed, since the early 1960s, experiments with plants and plant tissue cultures have been performed under various conditions of microgravity in space (one-way spaceships, biosatellites, space shuttles and parabolic flights, the orbital stations Salyut and Mir), accompanied by ground studies using rotating clinostat vessels (http://www.estec.esa.nl./spaceflights).

10.4 Plant Cell Cultures and Pharmaceuticals, and Other Biologically Active Compounds

Plant cells have been successfully used as “factories” to produce high-value secondary metabolites under economically viable conditions, in some notable cases. Since Tabata et al. (1974) first described the production of shikonin pigments by callus cultures of *Lithospermum erythrorhizon*, intensive efforts have been made to identify the regulatory factors controlling shikonin biosynthesis. As a result,

shikonin represents the first example of industrial production of a plant-derived pharmaceutical (Tabata and Fujita 1985). Shikonin is a red naphthoquinone pigment that is used in traditional dyes, another major application being for lipsticks. Shikonin acyl esters exhibit various pharmacological properties including anti-inflammatory and antitumor activity (Chen et al. 2002). Other examples are berberine production by cell cultures of *Coptis japonica*, rosmarinic acid production by cell cultures of *Coleus blumeii*, and sanguinarine production by cell cultures of *Papaver somniferum* (Eilert et al. 1985; Ulbrich et al. 1985). An example of a high-value drug produced partially from plant cell cultures is paclitaxel, an anti-cancer drug originally extracted from the bark of 50–60 year old Pacific yew trees (*Taxus brevifolia*; http://www.phyton-inc.com; Zenk et al. 1988; Ketchum et al. 1999; Tabata 2004). Recent advances in the molecular biology, enzymology, and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important secondary metabolites (Vanisree et al. 2004).

A brief description of some important secondary metabolites, their structure, and production in plant tissue culture is given below.

Alkaloids are a group of nitrogen-containing bases. They are physiologically active in humans (e.g., cocaine, nicotine, morphine, strychnine), and chemotherapeutics (vincristine, vinblastine, camptothecin derivatives, and paclitaxel). Some of the important alkaloids are nicotine of *Nicotiana*, the tropane alkaloids of *Hyoscyamus*, *Datura*, and *Atropa*, the isoquinoline alkaloids of *Coptis* and *Eschscholtzia californica*, and the terpenoid indole alkaloids of *Catharanthus roseus* and *Rauvolfia serpenitana* (Rates 2001; Hughes and Shanks 2002).

Papaver somniferum L. (opium poppy) is a traditional commercial source of codeine and morphine. Two tyrosine rings condense to form the basic structure of morphine. During this process, the first important intermediate is dopamine, which is also the starting substance of the biosyntheses of berberine, papaverine, and morphine. Production of morphine and codeine in morphologically undifferentiated cultures has been reported by Siah and Doran (1991).

Berberine is an isoquinoline alkaloid that occurs in roots of *Coptis japonica*, and the cortex of *Phellondendron amurense*. Berberine chloride is used for intestinal disorders in the Orient. However, it takes 5–6 years to produce *Coptis* roots as the raw material. Berberine has been reported from a number of cell cultures—e.g., *C. japonica*, *Thalictrum* spp., and *Berberis* spp. Sato and Yamada (1984) improved the productivity of berberine in cell cultures by optimizing the nutrients in the growth medium, and the levels of phytohormones.

L-DOPA, L-3,4-dihydroxyphenylalanine is the precursor of the alkaloids betalain, melanin, and others. It is also a precursor of catecholamines in animals, and is being used as a potent drug for Parkinson's disease, a progressive disabling disorder associated with a deficiency of dopamine in the brain. The widespread application of this therapy has created a demand for large quantities of L-DOPA at an economical price level, and this has led to the introduction of cell cultures as an alternative means for enriched production. Brain (1976) found that the callus tissue of *Mucuna pruriens*e accumulated 25 mg DOPA/l medium containing relatively

high concentrations of 2.4D. The DOPA synthesized by plant tissues is secreted mostly into the medium.

Scopolamine and hyoscyamine are tropane alkaloids that are used in anesthetic and antispasmodic drugs. Ornithine is one of the starting materials for their synthesis, and methylornithine is the first intermediate. These alkaloids occur in leaves of solanaceous plants including *Datura* sp., *Atropa*, *Hyoscyamus*, and *Scopolia* sp.

Capsicum frutescens produces the alkaloid capsaicin in nature, used as a pungent food additive largely in the eastern world. The sharp taste of the *Capsicum* fruit is caused by this substance. Suspension cultures of *C. frutescens* produce low levels of capsaicin. Yeoman and his group (Yeoman 1987) developed culture conditions for immobilizing the cells in reticulated polyurethane foam that could yield the same amounts of capsaicin as those obtained under natural conditions (see Sect. 3.3). M.A. Holden et al. (1988) reported elicitation of capsaicin in cell cultures of *C. frutescens* by spores of *Gliccladium deliquescens*. Biotransformation of externally fed protocatechuic aldehyde and caffeic acid to capsaicin in freely suspended cells and immobilized cell cultures of *C. frutescens* has also been reported (Rao and Ravishankar 2000). Jones and Veliky (1981) studied the effect of medium constituents on the viability of immobilized plant cells.

Withania somnifera Dunal (Solanaceae) is used as Indian ginseng in traditional Indian medicine. The active pharmacological components of *W. somnifera* are steroidal lactones of the withanolide type. Withanolides are known to have important pharmacological properties (antitumor, immunosuppressive), but they are also antimicrobial agents, insect deterrents, and ecdysteroid receptor antagonists. The principal withanolides in Indian *W. somnifera* are withaferin A and withanolide D. Both leaves and roots of the plant are used for the drug, and steroidal lactones occur in both parts. Ray and Jha (1999) reported production of withanolide D in roots transformed with *A. rhizogenes*, but withaferin A was not detected in the transformed root cultures, although both compounds are present in the leaves and roots of field-grown plants.

Steroids form a group of compounds comprising the sterols, bile acids, heart poisons, saponins, and sex hormones. Saponins constitute a group of structurally diverse molecules consisting of glycosylated steroids, steroidal alkaloids, and triterpenoids. However, one common feature shared by all saponins is the presence of a sugar chain attached to the aglycone at the C-3 hydroxyl position. The sugar chains differ substantially between saponins, but are often branched, and may consist of up to five sugar molecules (usually glucose, arabinose, glucuronic acid, xylose, or rhamnose). Sapogenins constitute the aglycone part of saponins, with well-known detergent properties. They are oxygenated C27 steroids with a hydroxyl group in C-3. Diosgenin is an example of these compounds.

Diosgenin is a saponin aglycone obtained from the roots of *Dioscorea* species. It is very similar to cholesterol, progesterone, and dehydroepiandrosterone (DHEA)—the precursor to testosterone. Diosgenin provides about 50% of the raw material for the manufacture of cortisone, progesterone, and many other steroid hormones, and is a multibillion dollar industry. The steroid synthesis pathway is cholesterol→pregnenolone→DHEA→testosterone→estrogen. However, the supply

of diosgenin cannot currently satisfy the demands of the ever-growing steroid industry, and therefore new plant species and new production methods, including biotechnological approaches, are being researched (Verpoorte 2000).

Several other groups have successfully obtained cell cultures for diosgenin production (Heble et al. 1967; Heble and Staba 1980; Jain et al. 1984; Huang et al. 1993). Kaul et al. (1969) studied the influence of various factors on diosgenin production by *Dioscorea deltoidea* callus and suspension cultures.

Dioscorea spp. (Dioscoreaceae) are frequently used as a tonic in traditional Chinese medicine, e.g., *Dioscorea doryophora*. Yeh et al. (1994) have established a cell suspension culture of *D. doryophora* Hance. Cell suspension cultures were obtained from microtuber- and stem node-derived callus in liquid culture medium supplemented with 0.1 mg 2.4D/l, 3% sucrose, and incubated in a rotary shaker at 120 rpm. Although 6% sucrose was found to be optimal for the growth of cell suspension culture, cells cultured in a 3% sucrose medium produced more diosgenin. Analysis by HPLC revealed that both stem node- and microtuber-derived suspension cells contained diosgenin. The microtuber-derived cell suspension culture contained 3.2% diosgenin per gram dry weight, the stem node-derived cultures only 0.3%. This is another example of influences of the origin of explants on the performance of cultured cells. As the amount of diosgenin obtained from a tuber-derived cell suspension is high, and similar to that found in the intact tuber (Chen 1985), a cell suspension culture can conveniently be used to produce diosgenin.

Cardenolides are naturally occurring glycosides that are widely distributed in plants. They are also called cardiac glycosides, because they exhibit the ability to strengthen the contraction of heart muscles. The best-known cardiac glycosides come from *Digitalis*, and include the drug digoxin. The aglycone is the non-sugar component of a glycoside molecule that results from hydrolysis of the molecule. Today, the sole source of the extensively used *Digitalis* drugs is the commercial harvesting of flowering *Digitalis* (foxglove) plants. The active compounds obtained from *Digitalis* include cardiac glycosides, digoxin, digitoxin, strophanthin, and ouabain. The structure of digitoxigenin is shown in Fig. 10.6, as a typical example of cardenolides.

A digoxin product, Lanoxin, is the brand name of a Burroughs Wellcome product, and has the largest market of the company's cardiovascular drugs. The major markets of Lanoxin are in the USA and Italy, and the total sales are approximately 6,000 kg per year at US$ 50 million. Other companies, such as Boehringer Mannheim, Merck Darmstadt, and Beiersdorf AG in Germany, also sell cardiac glycosides.

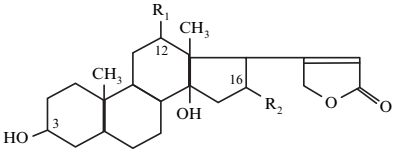


Fig. 10.6 Chemical structures of principal cardioactive glycosides of *Digitalis* species. Changes in R1 and R2 result in several new compounds

Digitalis lanata and *Digitalis purpurea* are commonly used for the production of cardiac glycosides (Fig. 10.6). Muir et al. (1954) were among the first to work in this field. Staba (1962) investigated the nutritional requirements of tissue cultures of *D. lanata* and *D. purpurea*.

10.4.1 Antitumor Compounds

Several antitumor compounds have been isolated from higher plants, but the concentrations of these active compounds in plants are generally low (Table 10.3). Some of the higher plant products, such as vinblastine, vincristine, podophyllotoxin derivatives including etoposide, and camptothecin and its derivatives, are marketed as very important anticancer drugs. Taxol, from *Taxus brevifolia* and related plants, is one of the most exiting compounds, and was marketed in 1992. Beside being controlled by the slow growth rate of these plants, the accumulation pattern of these compounds is dependent on geographical and environmental conditions. Large-scale harvesting of antitumor drug-yielding native plants is becoming a serious problem in terms of possible extinction, and steps are needed for environmental preservation.

Plants of the family Valerianaceae—e.g., *Nardostachys jatamansi*, *Valeriana wallichii*, and *Valeriana officinalis* L. var. *angustifolia*—have been used as folk medicines in India, Bhutan, and Nepal. *Nardostachys chinensis* has been employed in China for hundreds of years. These plants contain a group of compounds characterized by, e.g., sedative, tranquilization, cytotoxicity, and antitumor activities, and they are collectively called "valepotriates".

Becker and Chavadej (1988) induced callus tissues of nine different species of Valerianaceae on MS media, and found that *Fedia cornucopiae* and *Valeriana locusta* cells produced higher levels of the compounds than did the intact plants.

The dimeric terpenoid indole alkaloids, the anticancer drugs vincristine and vinblastine, are obtained from cultivated *Catharanthus roseus* (Apocyanaceae) plants. However, the process is not efficient, because of very low concentrations of

Table 10.3 Antitumor compounds isolated from higher plants

Antitumor compounds	Plant (dry wt. %)
Baccharin	2.0×10^{-2}
Bruceantin	1.0×10^{-2}
Camptothecin	5.0×10^{-3}
Ellipticine	3.2×10^{-5}
Homoharringtonine	1.8×10^{-5}
Maytansine	2.0×10^{-5}
Podophyllotoxin	6.4×10^{-1}
Taxol	5.0×10^{-1}
Tripdiolide	1.0×10^{-3}
Vinblastine, vincristine	5.0×10^{-3}

the alkaloids in the plant. It was reported that the concentration of both vinblastine and vincristine was only 0.0005% on a dry weight basis.

The vinblastine molecule is derived from two monomeric alkaloids, catharanthine and vindoline (Fig. 10.7a). The concentration of vindoline in the intact *C. roseus* plant is approximately 0.2% on a dry weight basis, which is much higher than that of catharanthine. The cost of vindoline is less than that of catharanthine and vinblastine.

Camptotheca acuminata, a native of northern China, was found to produce a potent antitumor alkaloid, camptothecin (Wall et al. 1966). In 2000, Wall obtained a US patent for a method of treating pancreatic cancer in humans with water-insoluble S-camptothecin of the closed lactone ring form, and derivatives thereof (Wall 2000). Sakato and Misawa (1974) induced *C. acuminata* callus on MS medium containing 0.2 mg 2.4D and 1 mg kinetin per liter, and developed liquid cultures in the presence of gibberellin, L-tryptophan, and a conditioned medium,

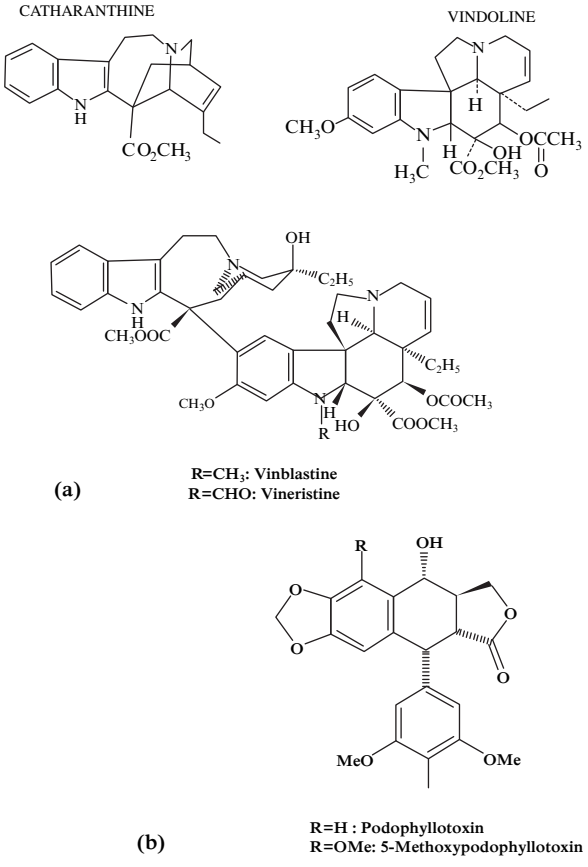


Fig. 10.7 (**a**) Chemical structures of catharanthine, vindoline, vinblastine, and vincristine. (**b**) Chemical structure of podophyllotoxin and 5-methoxypodophyllotoxin

which yielded camptothecin at about 0.0025% on a dry weight basis. In the cultures grown on MS medium containing 4 mg NAA/l, accumulation of camptothecin reached 0.998 mg/l (Van Hengel et al. 1992).

Podophyllotoxin is an antitumor aryltetralin lignan found in *Podophyllum peltatum* and *Podophyllum hexandrum*. It serves as a starting material for the preparation of its semi-synthetic derivatives, etoposide and teniposide, widely used in antitumor therapy of small-cell lung cancer, testicular cancer, acute lymphatic leukemia, and children's brain tumors (Issell et al. 1984). These slow-growing plants are collected from the wild, and are thus becoming increasingly rare. This limits the supply of podophyllotoxin, and necessitates a search for alternative production methods. Cell cultures of *P. peltatum* (Kadkade 1982), and *P. hexandrum* (Chattopadhyay et al. 2002) have been reported for production of podophyllotoxin (see also Arroo 2002).

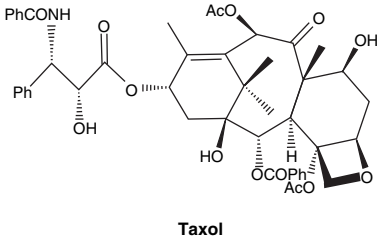
To increase the yield of podophyllotoxin, Woerdenbag et al. (1990) used a complex composed of a precursor, coniferyl alcohol, and b-cyclodextrin in *P. hexandrum* cell suspension cultures. Kadkade (1982) reported production of podophyllotoxin by *P. peltatum* cell cultures for the first time, and he found that a combination of 2.4D and kinetin in the medium yielded the highest production. Red light also stimulated the production. Since 5-ethoxypodophyllotoxin, an analogue of podophyllotoxin (Fig. 10.7b), has strong cytostatic activity, many researchers have tried to improve its yield through tissue cultures (e.g., Oostdam et al. 1993; see review by Ionkova 2007).

10.4.1.1 Taxol

Taxol, a diterpene amide obtained from *Taxus brevifolia*, is considered as the prototype of a new class of cancer chemotherapeutic agents. Some other plants, such as *Taxus canadensis* and *Taxus cuspidata*, also contain Taxol. The thin bark of the yew tree contains 0.001% Taxol on a dry weight basis. A century-old tree yields an average of 3 kg of bark, corresponding to 300 mg Taxol, which is approximately a single dose in the course of a cancer treatment. Due to low concentrations of Taxol in *Taxus*, the commercial production of Taxol poses a serious threat to these trees.

Pure Taxol was first isolated in 1969, and its chemical structure was disclosed in 1971 (Fig. 10.8; Wani et al. 1971). Taxol has a unique mode of action, because it stabilizes microtubules and inhibits depolymerization; consequently, cell division is inhibited at the M-phase of the cell cycle. Taxol is used for curing breast and lung cancer, and has shown positive results in curing ovarian cancer also. The FDA in the USA has approved Taxol (generically known as paclitaxel) at the end of 1992 for clinical treatment of ovarian and breast cancer.

The plant cell culture of *Taxus* sp. is also considered as one of the approaches available to provide a stable supply of Taxol and related taxane derivatives (Slichenmyer and Von Horf 1991). In 1989, Christen et al. reported for the first time the production of Taxol (paclitaxel) by *Taxus* cell cultures. They filed a US patent



Taxol

Fig. 10.8 Chemical structure of Taxol

describing that the tissue of *T. brevifolia* had been successfully cultured to produce Taxol-related alkaloids, and alkaloid precursors (Christen et al. 1991). Fett-Neto et al. (1995) have studied the effects of nutrients and other factors on paclitaxel production by *T. cuspidata* cell cultures (0.02% yield on dry weight basis). Srinivasan et al. (1995) have examined the kinetics of biomass accumulation and paclitaxel production by *T. baccata* cell suspension cultures. Paclitaxel was found to accumulate at high yields (1.5 mg/l) exclusively in the second phase of growth. Kim et al. (1995) established a similar level of paclitaxel from *T. brevifolia* cell suspension cultures, following 10 days in culture with optimized medium containing 6% fructose. Addition of carbohydrate during the growth cycle increased the production rate of paclitaxel, which accumulated in the culture medium (14.78 mg/l; Ketchum et al. 1999). Biotic and abiotic elicitors also improved the production and accumulation of Taxol through tissue cultures.

Factors influencing the stability and recovery of paclitaxel from suspension cultures and the media have been studied in detail by Nguyen et al. (2001). The effects of rare earth elements and gas concentrations on Taxol production have also been reported.

Shuler (1994) at Cornell University showed that a cell line of *T. brevifolia*, provided by the USDA Agriculture Research Station, and Phyton Catalytic, produced Taxol in the medium after 26 days in suspension culture. It is of interest that all the Taxol produced was secreted into the medium, which is very unusual for plant cell cultures.

Some other species of *Taxus* have been assessed for the production of Taxol. Vanisree et al. (2004) reported Taxol production from *Taxus mairei* calli induced from needle and stem explants on Gamborg's B5 medium supplemented with 2 mg 2.4D or NAA per liter. Different cell lines were established using stem- and needle-derived callus. One of the cell lines, after precursor feeding and 6 weeks of incubation, produced 200 mg Taxol per liter of cell suspension culture.

Ginseng

Ginseng (*Panax ginseng* C.A. Meyer), a classical herb widely used in East Asia, provides resistance to stress, disease, and exhaustion. Beveridge et al. (2002) have analyzed the phytosterol content in American ginseng seed oil. The root contains

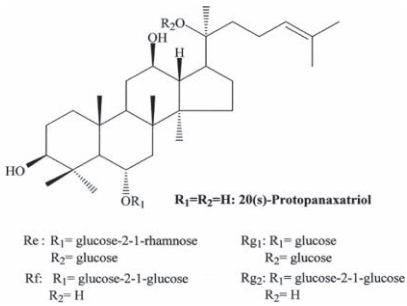


Fig. 10.9 Structure of ginsenosides, among which RG1 is the most important

various saponins and sapogenins. Among these, ginsenoside-Rb acts as a sedative, while Rg is stimulatory.

Recent progress in large-scale gene analysis (Jung et al. 2003), and proteome analysis (Kim et al. 2003; Nam et al. 2003) revealed that *P. ginseng* is one of the suitable sources for the study of dammarane-type triterpene saponin biosynthesis. This was the first result of molecular breeding to show the hyperaccumulation of triterpene saponins.

In recent years, ginseng cell culture has been explored as a potentially more efficient method of producing ginsenosides (Fig. 10.9). Medium components like carbon, nitrogen, and phosphate, potassium ion, and plant growth hormones influence the production of ginsenosides (Wu and Ho 1999; Zhang and Zhong 2004). Other types of tissue cultures, such as embryogenic tissues (Asaka et al. 1993) and hairy roots transformed by *Agrobacterium*, have been examined. Yu et al. (2002) reported ginsenoside production using elicitor treatments. These developments indicate that ginseng cell culture is still an attractive area for commercial development around the world, and it possesses great potential for mass industrialization.

Triterpenoids are a large class of natural isoprenoids present in higher plants that exhibit a wide range of biological activities. Changes in triterpenoid content during the growth cycle of cultured plant cells has been demonstrated (Kamisako et al. 1984).

The isoprenoid biosynthetic pathway plays an important role in plant metabolism. Sterols and triterpenes are widely distributed isoprenoids. Plant sterols, so-called phytosterols, have important pharmacological activities, including cholesterol-lowering and antitumor effects (Lee et al. 2004).

Ginkgo

Ginkgo produces important terpenoids. The root bark and leaves of *Ginkgo biloba* L. contain diterpenoids (ginkgolides) and a sesquiterpenoid (bilobalide) that have interesting pharmacological properties. Some studies have been made on undifferentiated cell cultures of *G. biloba* with the aim of producing ginkgolides in vitro. Enieux and Van Beekt (1997) studied ginkgolide in transformed and gametophyte-derived cell cultures of *G. biloba*.

10.4.2 Anthocyanin Production

Beside pharmaceutical compounds and food additives, perfumes and dyes have been produced in cultures of plant cells. Here, anthocyanin production serves as a model system to explain the basic mechanism of biosynthesis of secondary metabolites, their transport, and storage in plant tissue.

Anthocyanins are the large group of water-soluble pigments responsible for many of the bright colors seen in flowers and fruit. They are also used in acidic solutions in order to impart a red color to soft drinks, sugar confectionary, jams, and bakery toppings. The major source of anthocyanins for commercial purposes is grape pomaces, and wastes from juice and wine industries. Crude preparations of anthocyanins, which are relatively inexpensive, are used extensively in the food industry. The pure anthocyanins, however, are priced at US$ 1,250–2,000/kg.

Cell suspension cultures of *Vitis vinifera* produce anthocyanins after cessation of cell division (Kakegawa et al. 1995), and anthocyanin biosynthesis is regulated by the endogenous level of phenylalanine that is accumulated within the cells (Sakuta et al. 1994). Focusing on the fundamental understanding of the complex metabolic pathway, and regulation of secondary metabolism in plant cell cultures, Zhang et al. (2004) reviewed advanced knowledge of biosynthesis as well as post-biosynthesis pathways of anthocyanins from the genetic to the metabolite level. To illustrate this approach, they presented some data on the functional analysis of metabolic pathways for the biosynthesis of anthocyanins, from the profiling of gene expression and protein expression, to metabolic profiling in *Vitis vinifera* cell culture as a model system. Emphasis was placed on a global correlation at three molecular levels—gene transcript, enzyme, and metabolite, as well as on the interactions between the biosynthetic pathway and post-biosynthetic events that have been largely overlooked in earlier work (Zhang et al. 2004; Fig. 10.10).

End products of the flavonoid biosynthesis pathway include the anthocyanin pigments. Pigment extracts from plant sources generally contain mixtures of different anthocyanin molecules, which vary by their levels of hydroxylation, methylation, and acylation. The major anthocyanins (see Fig. 10.11) that accumulate in

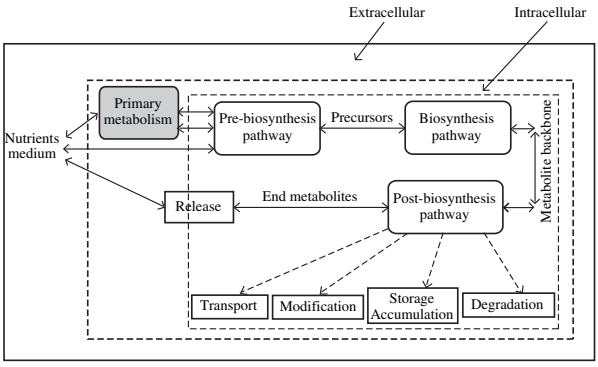


Fig. 10.10 Pathway events involved in the biosynthesis of a metabolite in plant cells: primary metabolism and secondary metabolism (pre-biosynthetic, biosynthetic, and post-biosynthetic pathways; after Zhang et al. 2004)

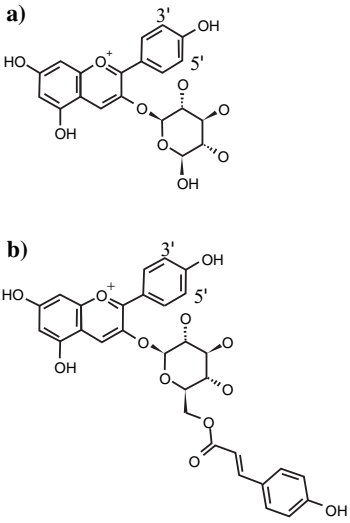


Fig. 10.11 Anthocyanin species present in *V. vinifera* suspension cultured cells: **a** 3-glucoside, **b** 3-p-coumaroylglucoside anthocyanin, and a summary table of modifications giving rise to the grape variants (Conn et al. 2003)

COMPOUND	3' Substitution	5' Substitution
Cyanidin	-OH	-H
Delphinidin	-OH	-OH
Peonidin	$-OCH_3$	-H
Petunidin	$-OCH_3$	-OH
Malvidin	$-OCH_3$	$-OCH_3$

V. vinifera cell culture are cyanidin 3-glucoside (Cy3G), peonidin 3-glucoside (Pn3G), malvidin 3-glucoside (Mv3G), and the acylated versions of these, cyanidin 3-p-coumaroylglucoside (Cy3CG), peonidin 3-p-coumaroylglucoside (Pn3CG), and malvidin 3-coumaroylglucoside (Mv3CG; Conn et al. 2003).

Anthocyanins are synthesized in the cytoplasm and transported into the vacuole, where they bind with a protein matrix and form anthocyanic vacuolar inclusions (AVIs; Fig. 10.12; Conn et al. 2003). AVIs were proposed to be the storage sites of anthocyanins. Anthocyanins assume their distinct color after transport to the vacuole, concomitantly diminishing feedback inhibition of cytosolic biosynthetic enzymes. Spherical pigmented inclusions are present in the vacuoles of specific cells in over 70 anthocyanin-producing species, and bind anthocyanins in a non-covalent manner (Markham et al. 2000). These insoluble protein matrices have been called anthocyanic vacuolar inclusions (Markham et al. 2000). It is thought

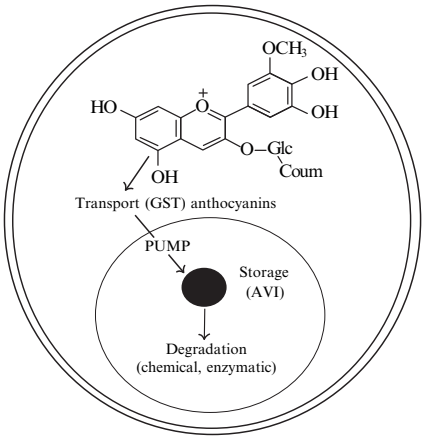


Fig. 10.12 Schematic summary of anthocyanin post-biosynthetic events. Anthocyanins are synthesized in the cytoplasm, and transported into the vacuole

that the anthocyanins are sequestered by AVIs primarily to increase their stability, but also to reduce inhibition of certain vacuolar enzymes (Conn et al. 2003).

The production of anthocyanins using cultured cells has been assessed in various plant species, and most studies use an anthocyanin-producing cell line as model system for secondary product production, because of the color that enables production to be easily visualized. Yamamoto et al. (1982), of Nippon Paint Co. in Japan, have studied the production of anthocyanins intensively. High osmotic potential in *Vitis vinifera* L. (grape) cell suspension cultures enhanced anthocyanin production. The addition of sucrose or mannitol in the medium increased the osmotic pressure, and the level of anthocyanins accumulated was increased. Similar observations were recorded on carrot cultures from our laboratory. The carrot secondary phloem explants grown on 4% sucrose produced excessive anthocyanins, compared to 2%, and were colored red (see Chap. 9; Kumar and Neumann, unpublished data; see also influences of micronutrients on anthocyanin production above, and Neumann 1962; Ozeki and Komamine 1986).

Saffron

Stamens of *Crocus sativus* give saffron, which is prized for use as a flavoring additive and as colorant. The stigma of the plant contains crocin (a yellow pigment), safranal (a fragrance), and picocrocin (a bitter substance). The plant is grown mainly in Spain and India, and about 30,000–35,000 handpicked blooms are required for the production of 1 lb of dry saffron. Crocin, being a glycoside, is water-soluble, and is not soluble in oils and fats. It is used in baked goods, soups, meat and curry products, cheese, confectionary, and as a condiment for rice in Indian foods. It also has medicinal value for stomach ailments.

Ajinomoto of Japan have attempted propagation of stigma-like saffron structures in vitro (Sano and Himeno 1987). They showed that crocin and picrocrocin were present, and after heat treatment (as done with field-grown stigmas), safranal was

produced. The composition of these phytochemicals corresponded with that of similarly treated, young intact stigmas (Himeno and Sano 1987).

Safflower yellow

Florets of the safflower plant (*Carthamus tinctorius* L.) give Mexican saffron or American saffron, a yellow pigment that has no relation to genuine saffron. The major pigment is carthamin, which exists at levels of up to 30% in the flowers, and there is also a red pigment in concentrations of about 0.5% (Wakayama et al. 1994).

Madder colorants

Rubia tinctorum (Rubiaceae) is a perennial plant, and its roots have been used as red dyes in Western Europe. The major components of the pigment are alizarin, purpurine, and its glycoside, ruberythric acid. Pure alizarin is an orange crystal soluble at 1 part to 300 in boiling water, and other solvents. Due to its high resistance to heat and light, it is suitable for the food industry. The callus of *R. tinctorum* induced from the root at San-Ei Chemical Industries of Japan was used to produce the pigment. After 21 days of cultivation in a 100-l jar fermenter, it produced approximately 1.5 g of the pigment (Odake et al. 1991).

10.5 Strategies for Improvement of Metabolite Production

As discussed at length above, cell cultures have been established from many plants, but often do not produce sufficient amounts of the required secondary metabolites (Rao and Ravishankar 2002). Nevertheless, in many cases the production of secondary metabolites can be enhanced by treating the undifferentiated cells with elicitors such as methyljasmonate, salicylic acid, chitosan, and heavy metals (DiCosmo and Misawa 1985; Barz et al. 1988; Gundlach et al. 1992; Ebel and Cosio 1994; Poulev et al. 2003). In some cases, secondary metabolites are produced only in organ cultures such as hairy root or shooty teratoma (tumor-like, see below) cultures; e.g., hairy roots produce high levels of alkaloids (Sevo'n and Oksman-Caldentey 2002), whereas shooty teratomas produce monoterpenes (Spencer et al. 1993).

In terms of cell growth kinetics, which usually incorporates an exponential curve phase, most secondary metabolites are produced during the stationary or plateau phase. This lack of production during the early stages can be explained by carbon allocation being mainly to primary metabolism during the active phase of growth. When growth stops, carbon is no longer needed in large quantities for primary metabolism, and secondary compounds are more actively synthesized. This is one explanation—others are possible (e.g., Chap. 12). It has been frequently observed that many new enzymatic activities, absent during the lag or log phases, appear

during the plateau phase. This has led many authors to propose a possible biochemical differentiation of the cells when growth stops (e.g., Payne et al. 1987; Charlwood et al. 1990). However, some secondary plant products are known to be growth-associated with undifferentiated cells, such as betalains and carotenoids.

The biosynthesis and accumulation of a number of secondary metabolites take place in specialized cells during specific developmental stage(s), i.e., differentiation and pigmentation in the organs and/or whole plants (Roja and Heble 1996).

10.5.1 Addition of Precursors, and Biotransformations

An exogenous supply of biosynthetic precursor to the culture medium, as discussed above, may increase the yield of the final product when productivity is limited by lack of precursor. The production of tropane alkaloids has been markedly increased by the addition of tropic acid, a direct precursor (see above; Tabata et al. 1971).

In contrast to the de novo synthesis, the biotransformation process with *Digitalis* plant cells seems to be more promising from a commercial point of view (see also Table 10.4, Figs. 10.13, 10.14). Graves and Smith (1967) reported that *D. lanata* and *D. purpurea* callus cultures rapidly transformed progesterone into pregnane.

Some cultures are able to transform cheap precursors into costlier chemicals. In the leaves of *Digitalis* are digoxin and digitoxin and their derivatives, where digitoxin is around one fourth of the concentration of digoxins. From a medical point of view, digoxin receives priority over digitoxin; the transformation of digitoxin

Table 10.4 Biotransformation of β-methyldigitoxin into β-methyldigoxin by *Digitalis lanata* cells in a 20-l reactor

Budget parameters	Weight	Proportion
B-Methyldigitoxin added	17.24 g	(100%)
Unconverted β-methyldigitoxin	2.04 g	(11.8%)
B-Methyldigoxin formed	14.36 g	(81.7%)
Byproduct	0.28 g	(1.4%)
Yield	94.90%	

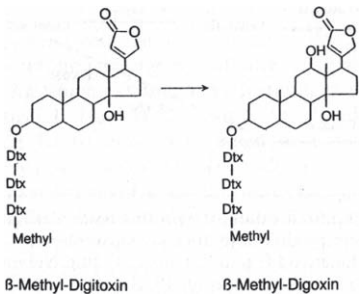


Fig. 10.13 Transformation of β-methyldigitoxin into β-methyldigoxin (Alfermann et al. 1985). *Dtx* Digitoxose

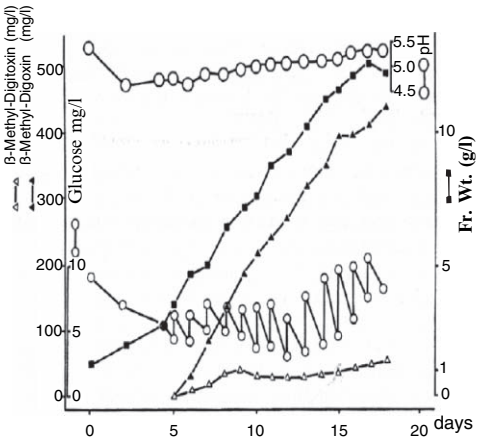


Fig. 10.14 Biotransformation of β-methyldigitoxin into β-methyldigoxin by cell cultures of *Digitalis lanata* in a 200-l airlift reactor (Alfermann et al. 1985)

into digoxin has been carried out successfully in undifferentiated cultures. The reaction takes place by attachment of a hydroxyl group in C-12 of the digoxins, and these "wastes" of digitoxin production can be utilized by the pharmaceutical industry (Alfermann et al. 1983, 1985; Figs. 10.13, 10.14).

Digitoxin is diverted into different products. One of this is methyldigitoxin. Certain cell clones have been isolated that transform methyldigitoxin into methyldigoxin (Figs. 10.13, 10.14). For industrial usage, generally digoxin obtained from the leaves is methylated through a chemical process.

At the beginning of the experiment, the substrate is fed into the fermenter, and after 13 days about 70% of methyldigitoxin is transformed into methyldigoxin, and is excreted to the nutrient medium; 20% could be located in cells in the form of digitoxin and digoxin, and some remains of the substrates were still in the nutrient medium. Using this technique, within 2 weeks of experimentation 430 mg methyldigoxin was produced in a 200-l fermenter culture. This approach was developed further into a semi-continuous process in which a relatively long "scale up period" is reduced to 17 days, and thus the cost of production is reduced. Here, after 14 days only 85% of the contents of the fermenter is taken for the extraction, and the rest remains as inoculum for the next culture. The fermenter is provided with fresh nutrient medium, and by providing the substrate the manufacture of digoxin could be achieved afresh. This subculture was employed six times without loss of transformation capacity. During the 3 months of investigation, 500 g of methyldigoxin was produced, and based on a report from Alfermann et al. (1985), this would suffice to treat 1,000 patients for more than 7 years.

Such "biotransformations" are also possible in other systems where the chemically synthesized compounds are obtained from cell cultures of either the same or another plant. One expects new, unknown molecules produced to replace economically important chemical substances.

Normal root cultures of *Capsicum frutescens* biotransform externally fed precursors like caffeic acid and veratraldehyde into vanillin and other related metabolites.

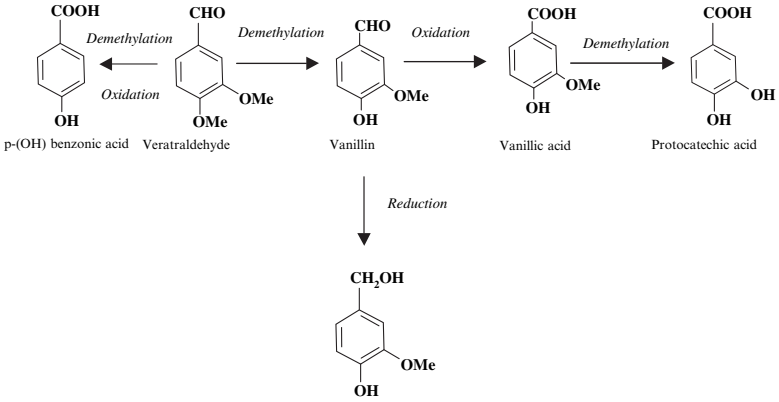


Fig. 10.15 Probable biosynthetic pathway of the vanilla-flavored metabolite veratraldehyde via biotransformation (after Suresh and Ravishankar 2005)

The bioconversion of caffeic acid into further metabolites—viz. vanillin, vanillylamine, vanillic acid—was shown to be elicited by treating the cultures with 10 μM methyljasmonate (Suresh and Ravishankar 2005). Root cultures treated with MeJa accumulated 1.93 times more vanillin (20.2 μM on day 3) than did untreated ones. Among all the precursors studied for the biotransformations, *Capsicum* root cultures could biotransform veratraldehyde most efficiently, leading to highest production of vanillin (78 μM on day 6 after veratraldehyde addition) than for any other phenylpropanoid precursor. The probable biosynthetic pathway of veratraldehyde biotransformation is indicated in Fig. 10.15, which shows that the precursor veratraldehyde would be enzymatically demethylated to vanillin and parahydroxybenzoic acid. Upon oxidation, vanillin would be converted into vanillic acid, which in turn would be demethylated to yield protocatechuic acid. The formation of vanillyl alcohol would be by reduction of vanillin.

10.5.2 Immobilization of Cells (see also Sect. 3.3)

In immobilization (Yeoman 1987; Holden and Yeoman 1987), plant cells or microaggregates are encapsulated in polymers (alginate, carraghenans, etc.), and this usually enhances the production of secondary metabolites (Gontier et al. 1994). The main explanations for this come from a possible matrix effect of the polymers around the cells, which could mimic the tissue organization between these. This reportedly gives rise to the so-called biochemical differentiation that favors the synthesis of secondary products (Yeoman 1987; Gontier et al. 1994).

Biotransformation of codeinone into codeine with immobilized cells of *Papaver somniferum* has been reported by Furuya et al. (1972). The conversion yield was 70.4%, and about 88% of the codeine converted was excreted into the medium.

Ishida (1988) established *Dioscorea* immobilized cell cultures in which reticulated polyurethane foam was shown to stimulate diosgenin production, increasing the cellular concentration by 40% and the total yield by 25%.

10.5.3 Differentiation and Secondary Metabolite Production

Differentiated cell cultures are reported to have a higher biochemical potential (Yeoman and Yeoman 1996). There is a tight link between morphological differentiation, and differentiation in metabolite biosynthesis in plant cells. Characterization of such metabolic differentiation at the molecular level is an important step in the development of effective methods to induce high levels of secondary metabolite production in cultured plant cells (Krisa et al. 1999). As an example, established hairy root cultures following infection with *Agrobacterium rhizogenes* displayed an enhanced production of those secondary metabolites that occur naturally in untransformed roots, resulting in amounts of secondary compounds comparable to, or even higher than those present in intact roots (Sharp and Doran 1990; Zárate 1999).

In some cases, complete differentiation may not be required, and tissue differentiation could increase secondary metabolite production. As an example, the development of xylem differentiation in calli of *Duboisia myoporoides* R. Br. led to the expression of stable tropane alkaloid biosynthesis without the need to regenerate differentiated organs. This finding may enable commercial tropane alkaloid production from calli with differentiated xylem, but not requiring organ development (Khanam et al. 2000).

Although cell cultures have been attempted from several *Digitalis* spp., those obtained from *Digitalis lanata* are most extensively utilized. As with the other systems, the undifferentiated callus culture is not able to produce the secondary metabolism-based metabolite glycosides. This has been demonstrated using highly sensitive RIA tests. Others, like the steroid testosterone, were detected using sensitive analytical methods. The glycosides were detectable as soon as the process of differentiation started (Luckner and Diettrich 1985, 1987). Such differentiation could be either the formation of compact green globuli in cell suspensions, shoot differentiation, or differentiation of embryos. These different types of differentiation patterns are associated with variations in the auxin/cytokinin ratio in the nutrient medium. In the green globuli, the cardenolid concentration was very low, i.e., 0.01 mg per g dry matter. In unspecialized cell cultures, or in the dark, the concentrations were even lower (less than 0.001 mg/g f. wt.). Light can increase the cardenolidic concentrations, though light is not essential for the biosynthesis of cardenolides. This is only of quantitative importance here (Luckner and Diettrich 1985, 1987). Induction of embryogenesis in these cultures significantly enhanced the cardenolide concentrations (0.7 mg/g dry matter). The concentration in the shoot apex was somewhat lower, at 0.4 mg/g dry matter. Thus, morphogenesis induced higher concentrations of secondary metabolites in general.

The carbohydrate and nitrogen composition of the medium also influences the production of cardenolides. Maltose was found to be most suitable. It is composed of two molecules of glucose as a disaccharide. Sucrose, and also both the monosaccharides glucose and fructose lead to lower levels of embryogenic differentiation, and also lower levels of cardenolide concentration. Also the source of nitrogen supply influences embryogenesis, and the cardenolide concentration; the optimum for both processes is achieved through a mixture of ammonium and nitrate in ratios of 1:5–1:10. By contrast, glutamine, and ammonium salts of different organic acids have negative effects.

In different experiments to isolate high-yielding strains from suspension cultures based on the selection of cardenolide levels, a broad variability in cardenolide concentration was documented in the colonies. In such cultures, variations in cardenolide concentrations are not due to genetic factors, but rather to the developmental stages of the cultures (Luckner and Diettrich 1985).

The synthesis of the tropane alkaloids hyoscyamine and scopolamine in *Atropa belladonna* and *Catharanthus roseus* is developmentally regulated, the highest levels occurring in younger, faster dividing regions of the plant (De Luca and St-Pierre 2000). Physical factors that influence differentiation also affect secondary metabolite production; e.g., light has been reported to be necessary for the synthesis of vindoline, an important precursor of vincristine and vinblastine in *C. roseus* (De Luca and St-Pierre 2000). The pathways of interest often involve multiple organellar compartments, resulting in transport limitations and sequestered pools of metabolites. The lack of differentiation in cell cultures has often also been a barrier to successful alkaloid production. As an example, the synthesis of strictosidine, the precursor to the indole alkaloids, requires three organellar compartments. Tryptophan and the terpenoid precursor geraniol are synthesized in the plastids, tryptophan is then decarboxylated in the cytosol, and the two moieties are condensed in the vacuole (De Luca and St-Pierre 2000).

Duboisia myoporoides R. Br., an Australian member of the Solanaceae family, contains different groups of alkaloids, and is cultivated in Australia for its high scopolamine content. In the complete *D. myoporoides* plant, alkaloid biosynthesis takes place in the root cells (Hashimoto and Yamada 1994). While different classes of alkaloids have been detected in cultured roots of this species (Yukimune et al. 1996; Khanam et al. 2000), tropane alkaloids are found in the cultured shoot only after root initiation (Kukreja et al. 1986; Lin and Tsay (2004).

Alkaloid production has also been studied in *Corydalis ambigua* (Papaveraceae). Corydaline and cavidine were accumulated in the leaf, tuber, and somatic embryos, whereas corybulbine was detectable only in tubers. Callus cultures and immature seeds, which lack embryos, contain only trace amounts of these alkaloids, suggesting the necessity of organ differentiation for alkaloid production in *C. ambigua*. Somatic embryos of *C. ambigua* that were cultured in liquid Linsmaier and Skoog medium, supplemented with 0.1M IAA and 3% sucrose, produced two tetrahydroprotoberberine alkaloids, corydaline (0.03% of dry cell weight), and cavidine (1.09%; Hiraoka et al. 2004). These investigations show that the differentiation status can increase the cardenolide concentration. Still, the yield lies below the level of profitable commercial utilization.

10.5.4 Elicitation

Elicitation is usually one of the most successful strategies to increase secondary metabolite production. This consists in applying chemical or physical stresses to the cell suspension cultures that trigger the production of secondary metabolites normally not produced, or only at low concentrations. Elicitors are defined as molecules that stimulate defense or stress-induced responses in plants (Van Etten et al. 1994). Elicitors like jasmonade and its derivatives are known to stimulate the production of secondary metabolites in plants (Sanz et al. 2000).

Beside enhancing the production of some of the desired secondary metabolites, elicitor treatment also activates the genes involved in the biosynthesis of such compounds. This has been recently demonstrated in studies targeting nicotine biosynthesis in tobacco cells (Breyne and Zabeau 2001; Goossens et al. 2003a).

Furanocoumarins, the phytoalexins having medicinal and industrial value, are restricted to the four families Leguminosae, Apiaceae, Umbelliferae, and Rutaceae. As their name suggests, these compounds are based on a skeleton formed by a furan ring fused to a coumarin unit. At present, furanocoumarins are produced from essential oils of bergapten (*Citrus bergamia*), but this production is insufficient to meet the increasing demands for these molecules. Elicited response was demonstrated very clearly for cell cultures of *Ruta graveolens* exposed to autoclaved culture homogenate of *Rhodoturula rubra*. The increased accumulation of these compounds was preceded by induction of specific secondary-product enzymes like L-phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), S-adenosyl-L-methionine: bergaptol-O-methyltransferase (BMT), and S-adenosyl-L-methionine: xanthtoxol-O-methyltransferase (XMT). The enzymes showed sequential changes in activity with time for PAL and 4CL (Diwan and Malpathak 2007).

10.5.4.1 Jasmonic Acid

Jasmonic acid (JA), and its methyl ester (MJ) have also been studied as elicitors of secondary metabolites in plants with hairy roots (Rao and Ravishankar 2002). Exogenous MJ has been shown to mimic the effects of wounding through the induction of proteinase inhibitors (Xu et al. 1993), vegetative storage proteins (Berger et al. 1995), and secondary metabolites such as nicotine (Baldwin et al. 1994). MJ fed along with fungal elicitors was reported to activate the enzyme phenylalanine ammonia lyase (PAL), resulting in higher production of scopoletin and scopoline in tobacco cell cultures (Sharan et al. 1998), taxol accumulation in cell suspension cultures of *Taxus chinensis* (Wu and Lin 2003), and ginsenoside production by cell suspension cultures of *Panax ginseng* in 5-l balloon-type bubble bioreactors (Thanh et al. 2005). It was also shown that MJ induces the expression of polyphenol oxidase (ppo) genes, and markedly increases the level of the enzyme. Hayashi et al. (2003) studied upregulation of soyasaponin biosynthesis by methyljasmonate in cultured cells of *Glycyrrhiza glabra*.

Increased levels of enzymes induced by MJ promoted the formation of secondary metabolites for a broad range of plant species (Blechert et al. 1995). In *Catharanthus roseus* (Madagascar periwinkle), methyljasmonate induces terpenoid indole alkaloid (TIA) production. ORCA (octadecanoid-responsive catharanthus AP2/ERF domain) transcription factors have been shown to regulate the JA-responsive activation of several TIA biosynthesis genes (Endt et al. 2002; Memelink and Gantet 2007).

A promoter element involved in jasmonate- and elicitor-responsive gene expression (JERE) was identified in the TIA biosynthetic gene strictosidine synthase (STR; Menke et al. 1999).

Interaction of MJ, wounding, and fungal elicitation influenced the production of sesquiterpenes in *Agrobacterium*-transformed root cultures of *Hyoscyamus muticus* (Choi et al. 2001, 2005). These results indicate that signaling, in addition to MJ, is required for the induction of these phytoalexins.

Jasmonic acid also altered the accumulation of major anthocyanins in *Vitis vinifera* cell cultures (see above). Peonidin 3-glucoside content at day 3 was increased from 0.3 to 1.7 mg/g dry cell wt., while other major anthocyanins were increased less. Light further enhanced anthocyanin accumulation induced by jasmonic acid elicitation (Curtin et al. 2003).

MJ treatment increases the levels of ginsenoside. Choi et al. (2005) analyzed the ESTs (expressed sequence tags) derived from MJ-treated ginseng hairy roots, and attempted to identify the genes involved in the MJ-induced biosynthesis of various secondary metabolites, including ginsenosides.

Hypericum perforatum L. (St. John's wort) produces hypericin, a photosensitive napthodianthrone considered to be responsible for the reversal of depression symptoms. Production levels, and localization of hypericin in cell suspension cultures are entirely different from those of an intact plant (Bais et al. 2002).

JA elicitation of *Hypericum* cells increased the accumulation of phenylpropanoids and naphtodianthrones (Gadzovska et al. 2007). Earlier, Walker et al. (2002) reported that an administration of 250 mM JA induced an increased accumulation of hypericin in cultured cells of *H. perforatum* L. grown under dark conditions (0.318–0.02 mg/g dry wt.), compared to JA-elicited cultures under light conditions (0.089–0.006 mg/g dry wt.), and their respective controls (Bais et al. 2002). It is likely that secondary metabolites, in particular phenolic compounds, can constitute a photoblock resulting in hindered photoconversion under continuous light conditions (Hahlbrock and Scheel 1989).

10.5.4.2 Effect of UV on Production of Secondary Metabolites in Cultured Tissues

Resveratrol and piceatannol have various beneficial health effects—e.g., moderate intake of resveratrol or resveratrol-containing food, such as red wine, may diminish the risk of cardiovascular diseases (Maxwell et al. 1994). Many studies have linked the antitumor activities of resveratrol and piceatannol to their abilities to inhibit cell proliferation, and arrest cells in the S-phase (Jang et al. 1997; Joe et al. 2002).

Several research groups have demonstrated the ability to produce stilbenoid compounds from cultured plant tissues under normal or induced conditions. For example, resveratrol has been isolated from suspended cell cultures of grape, *Vitis vinifera*, and peanut, *Arachis hypogaea* (Schöppner and Kindl 1984; Ku et al. 2005). In the callus of *A. hypogaea*, isopentenyl resveratrol was induced via UV irradiation (Fritzemeier et al. 1983).

10.6 Organ Cultures

Plant organ cultures represent an interesting alternative to cell cultures for the production of plant secondary products. Two types of organs are generally considered for this purpose: hairy roots, and shoot cultures (Fig. 10.16).

10.6.1 Shoot Cultures

Shoot cultures are often superior generators of secondary metabolism products than are callus or suspension cultures. Although no trace of the dimeric alkaloids anhydrovinblastine and leurosine were detected in suspension cultures of *C. roseus*, these have been detected in shoot cultures. Similarly, much higher levels of quinine and related alkaloids are present in shoot cultures than in suspension cultures of *Cinchona ledgeriana*. More cardiac glycosides are accumulated in shoot cultures of *Digitalis* than in undifferentiated cultures of this species. To date, however, techniques for shoot cultures are underdeveloped for mass cultivation. Nevertheless,

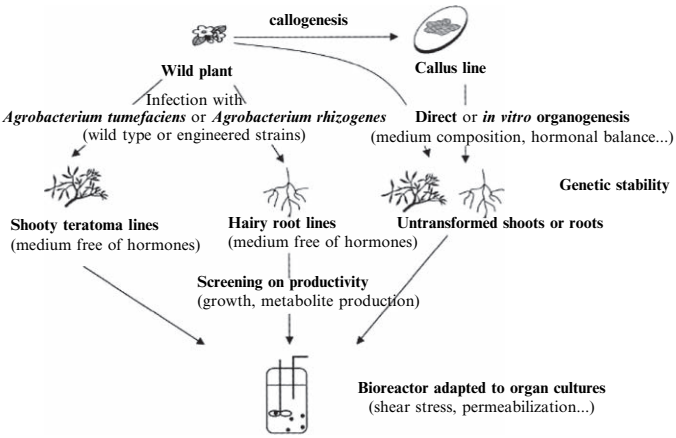


Fig. 10.16 Guidelines for the production of secondary metabolites from plant organ cultures (after Bourgaud et al. 2001)

there is a promising possibility to produce products associated specifically with shoots or leaves. Shoot cultures can easily be established simply by removing the top from sterile seedlings, and placing it in B5 or MS media.

10.6.2 Root Cultures

Until recently, only a very limited number of plant species had been recorded for yield in root cultures, which could be grown indefinitely with an acceptable growth rate. The possibility of genetic transformation for root culture has been mentioned earlier. *Agrobacterium rhizogenes* are soil-borne bacterial pathogens of plants, and these bacteria can enter into any wounded part of plant cells (Sect. 13.2). The Ri plasmid of *A. rhizogenes* induces rhizogenesis in inoculated cells of numerous roots. Such roots can be cultured, and unlike undifferentiated culture cells, stably maintain the biosynthetic characteristics of the original plant. This system has therefore been used as a means of culturing cells that will synthesize and accumulate secondary metabolite characteristics of the roots of the intact plant.

Based on this technique, hairy root cultures have already made an impact on the production of certain secondary metabolites. Considerable increases in biomass and alkaloid accumulation in root cultures can be achieved by RI-TDNA. Jung and Tepfer (1987) reported that transformation improved root growth in agitated flasks, and these roots produced tropane alkaloids at levels similar to those recorded for roots of the corresponding intact plants.

Hairy roots, the result of genetic transformation by *Agrobacterium rhizogenes*, have attractive properties for secondary metabolite production (Fig. 10.16; Park and Facchini 2000; Kim et al. 2002; Pavlov and Bley 2005). In some cases, secondary metabolites are produced only in organ cultures, such as hairy root or shooty teratoma (tumor-like) cultures. For example, hairy roots produce high levels of alkaloids (Sevo'n and Oksman-Caldentey 2002).

The greatest advantages of hairy roots is that they often exhibit about the same, or greater biosynthetic capacity for secondary metabolite production as do their mother plants, and are able to grow on growth regulator-free media. Many valuable secondary metabolites are synthesized in roots in vivo, and often synthesis is linked to root differentiation (Flores Berrios et al. 2000). Even in cases where secondary metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the metabolites as well. For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of *Lawsonia inermis* grown in half- or full-strength MS medium (Table 3.3) can produce lawsone under dark conditions (Bakkali et al. 1997). Likewise, artimicin accumulates only in the aerial part of *Artemisia annua* plants, but several laboratories have shown that hairy roots can produce artemisinin (Liu et al. 1997). Genetic stability is another characteristic of hairy roots.

Hairy root cultures of the endangered species *Atropa baetica* display high accumulation of the major tropane alkaloids, atropine (±hyoscyamine) and scopolamine,

with atropine levels similar to those of intact non-transformed roots. Surprisingly, scopolamine levels were fourfold higher than for intact roots, suggesting a much higher H6H activity (hyoscyamine 6-β-hydroxylase; Hashimoto and Yamada 1994; Zárate et al. 2006), this being the enzyme responsible for the conversion of hyoscyamine into scopolamine.

Catharanthus roseus hairy roots, in a fast-growing, differentiated tissue culture generated by *Agrobacterium rhizogenes* infection, accumulated higher levels of alkaloids than was the case for undifferentiated cell and callus cultures (Moreno-Valenzuela et al. 1998). The biosynthesis of vindoline was reported to be significant only in shooty teratomas (O'Keefe et al. 1997), or shoots regenerated from calli (Miura et al. 1988). Shimomura et al. (1991) established a hairy root culture of *Lithospermum erythrorhizon* with *A. rhizogenes*. The hairy root culture did not produce shikonin on solid MS medium, but did produce the pigment in the root culture medium, and also secreted it into the medium.

Solasodine present in Solanaceae plants has gained significant importance globally. Certain fast-growing hairy root clones of *Solanum khasianum* are reported to be high producers of solasodine (Aird et al. 1988). The effect of nitrogen on growth and solasodine production, when nitrate and ammonia are used as nitrogen source, has been demonstrated by Jacob and Malpathak (2005).

10.7 Genetic Engineering of Secondary Metabolites (see also Sect. 13.2)

Production of secondary metabolites is under strict regulation in plant cells, due to coordinate control of the biosynthetic genes by transcription factors. Transcription factors are involved in secondary metabolism, and their role has been reviewed by Endt et al. (2002). Several transcription factors involved in the regulation of alkaloid biosynthesis genes have been isolated and studied. There are indications that the abundance and activities of transcription factors per se are regulated by external signals (Endt et al. 2002; Memelink and Gantet 2007).

Based on this success, genetic transformation of medicinal plants has been attempted, primarily to enhance the production of various pharmaceuticals, but also flavors and pigments. The potential of metabolically engineered plant-derived secondary metabolites is high, and has been well documented by modifying anthocyanin and flavonoid pathways, leading to changes in flower color, or increased levels of antioxidative flavonol production in tomato (Muir et al. 2001). To date, however, there has been little success in modifying pathways to form pharmaceutically important compounds. Transgenic cultures and plants have been reported some time ago for about 70 species (Bajaj and Ishimura 1999). Hashimoto et al. (1993) reported increased production of tropane alkaloids in genetically engineered root cultures. There are several strategies that can be used to enhance the production of desired pharmaceuticals by genetic engineering (Veerporte et al. 2000; Sumner et al. 2003). Oksman-Caldentey and Inzé (2004) have reviewed the work on the production of designer metabolites in the post-genomic domain.

Functional genomics approaches (transcriptomics, proteomics, and metabolomics) are powerful tools for accelerating comprehensive investigations of biological systems (Fig. 10.17). Because no genomic tools are available for most plants producing interesting secondary metabolites (e.g., terpenoid indole alkaloids, and paclitaxel), it is not surprising that virtually no such comprehensive studies have been reported yet.

Recent years have witnessed a true revolution in the profiling of primary, but also secondary metabolites (Fiehn et al. 2001). Several genes in the biosynthetic pathways for scopolamine, nicotine, and berberine have been cloned, making the metabolic engineering of these alkaloids possible (Fig. 10.18). Expression of two

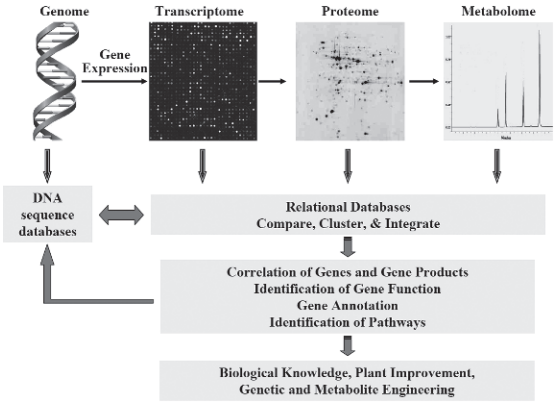


Fig. 10.17 Functional genomics approaches (transcriptomics, proteomics, and metabolomics) are powerful tools for accelerating comprehensive investigations of biological systems (Yeoman et al. 1980)

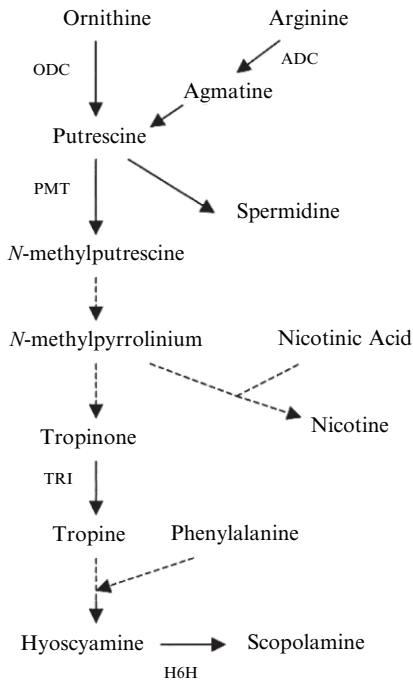


Fig. 10.18 Cloned genes in the nicotine and tropane alkaloid pathways. *ODC* Ornithine decarboxylase, *ADC* arginine decarboxylase, *PMT* putrescine N-methyltransferase, *TRI* tropinone reductase I, *H6H* hyoscyamine 6-β-hydroxylase (after Hughes and Shanks 2002)

branching-point enzymes was for engineered putrescine N-methyltransferase (PMT) in transgenic plants of *Atropa belladonna* and *Nicotiana sylvestris*, and (S)-scoulerine 9-O-methyltransferase (SMT) in cultured cells of *Coptis japonica* and *Eschscholzia californica*. Overexpression of PMT increased the nicotine content in *N. sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content, and induced abnormal morphologies. Ectopic expression of SMT caused the accumulation of benzylisoquinoline alkaloids in *E. californica*. However they explore solutions to such challenges, metabolic engineers must nevertheless take care in recognizing the limitations inherent in designing plant systems (Hughes and Shanks 2002).

Based on the positive correlation between PMT activity and nicotine synthesis, Sato et al. (2001) expressed tobacco PMT using the CaMV 35S promoter in *N. sylvestris* plants. In the overexpressing lines, a 40% increase in nicotine content was noted over controls.

Another example is the observation that antisense-mediated downregulation of putrescine N-ethyltransferase in transgenic tobacco plants resulted in a concomitant reduction in nicotine content, but surprisingly also in elevated levels of the secondary metabolite anatabine (Chintapakorn and Hamill 2003). These examples, as well as others comprehensively reviewed elsewhere (Sato et al. 2001; Verpoorte and Memelink 2002; Hashimoto and Yamada 2003; Magnotta et al. 2007), show that engineering a single functional gene has considerable value for metabolic engineering, but also some limitations. The ability to switch on entire pathways by ectopic expression of transcription factors suggests new possibilities for engineering secondary metabolite pathways.

The first step in approaching the engineering of the pathways has been an attempt to quantify the relative importance of the terpenoid and indole pathways with precursor feedings. Although the terpenoid pathway has generally been found to be limiting in hairy root and cell cultures (Morgan and Shanks 2000), a few cell lines responded to indole feeding (Whitmer et al. 1998). Results for hairy roots also demonstrate that growth stages play a key role in determining the relative importance of the two pathways (Morgan and Shanks 2000). Thus, tryptophan decarboxylase (TDC) activity coincides with alkaloid accumulation, while strictosidine synthase (STR) activity is relatively stable (Meijer et al. 1993a, b).

In manipulating the alkaloid contents of *Cinchona officinalis*, TDC overexpression was ineffective. TDC has, however, proved useful in other systems, like canola, as a means of diverting flux to a metabolic sink to reduce undesired products (Chavadej et al. 1994). It has also been used to manipulate the alkaloid contents of *C. officinalis* (Geerlings et al. 1999).

Although metabolic engineering of alkaloid production is still in its infancy, the field offers great promise. Other papers have extensively reviewed the strategies of engineering pathways, methods for cloning genes; means to quantify flux, tools to characterize pathways at the enzymatic level, and techniques for pathway elucidation at the metabolite level (Morgan et al. 1999; Ratcliffe and Shachar-Hill 2001).

Triterpene saponins are important bioactive compounds in many other medicinal plants—for example, glycyrrhizin in *Glycyrrhiza* sp. (Dixon and Summer 2003),

and saikosaponins in *Bupleurum falcatum* (Aoyagi et al. 2001). Squalene synthase (SS; EC 2.5.1.21) catalyzes the first enzymatic step from the central isoprenoid pathway toward sterol and triterpenoid biosynthesis (Abe et al. 1993).

From EST data analysis, Devarenne et al. (2002) identified several genes involved in the biosynthesis of 2,3-oxidosqualene, such as SS and SE that are upregulated in MJ-treated hairy roots. SS is involved in the biosynthesis of squalene from farnesyl diphosphate. This reaction is the first step in the transfer of carbon from the isoprenoid pathway toward triterpene biosynthesis, and may be a potential point of triterpene biosynthesis regulation (Devarenne et al. 2002). SE converts squalene into 2,3-oxidosqualene, which serves as the substrate for the synthesis of protopanaxadiol. Devarenne et al. (2002) also identified three transcripts encoding SE, which suggests that SE forms a small multigene family in the ginseng genome. Moreover, they identified genes encoding two 3-hydroxy-3-methylglutaryl CoA reductases (HMGR), and a farnesyl diphosphate synthase (FPS). These enzymes are considered to constitute potential regulatory points in the isoprenoid pathway (Devarenne et al. 2002). Most of the ginseng genes described above, which were identified on the basis of their function in other plants, had never previously been identified. The first committed step in the biosynthesis of the triterpenoid saponin, ginsenoside, involves the cyclization of oxidosqualene by oxidosqualene cyclase (OSC), which produces one of several different triterpenoids, including protopanaxadiol (Figs. 10.19, 10.20).

10.8 Membrane Transport and Accumulation of Secondary Metabolites

Plants produce a large number of secondary metabolites, and their subcellular localization is highly regulated according to their biosynthetic routes and structural features. To achieve their function, such as protection against UV light or pathogens, they are generally accumulated in specific tissues or cell types. Some examples of secondary metabolite production and storage are given in Table 10.5.

Secondary metabolites are often transported from source cells to neighboring cells, or even further to other tissues or remote organs (Kunze et al. 2002). Yazaki (2005) studied transporter proteins for these natural products in plants. Storage vacuoles, which often occupy 40–90% of the inner volume of plant cells, play a pivotal role in the accumulation of secondary metabolites in plants (Fig. 10.21). The accumulation of secondary metabolites in vacuoles has at least two positive roles: the sequestration of biologically active endogenous metabolites inside the cells, and the protection of such metabolites from catabolism (Gunawardena et al. 2004). Two major mechanisms are proposed for the vacuolar transport of secondary metabolites, these being H^+ gradient-dependent secondary transport via the H^+-antiport, and directly energized primary transport by ATP-binding cassette (ABC) transporters (Martinoia et al. 2002).

Membrane transport is fairly specific and highly regulated for each secondary metabolite, and recent progress in genome and expressed sequence tag (EST)

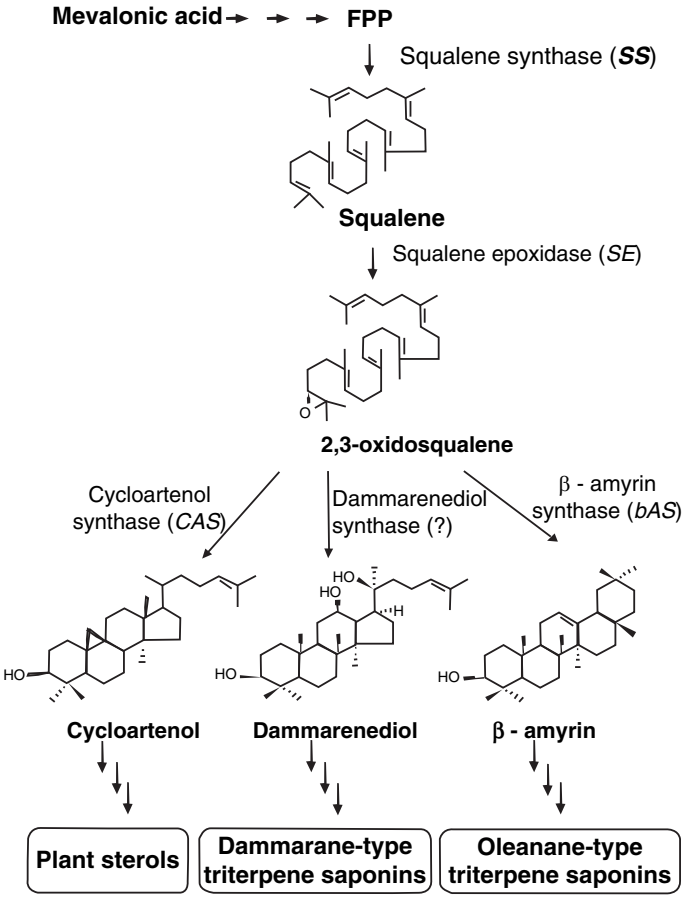
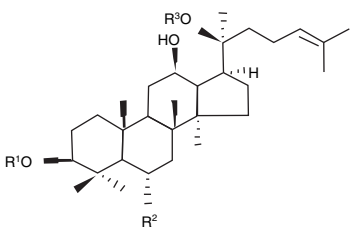


Fig. 10.19 Metabolism of squalene

Dammarane-type triterpene saponins



	R^2	R^1	R^3
Ginsenoside Rb_1	-H	- Glc^2-Glc	-Glc^6-Glc
Ginsenoside Rb_2	-H	- Glc^2-Glc	-Glc^6-Ara(pyr)
Ginsenoside Rc	-H	- Glc^2-Glc	-Glc^6-Ara(fur)
Ginsenoside Rd	-H	- Glc^2-Glc	-Glc
Ginsenoside Re	-O-Glc^2-Rha	-H	-Glc
Ginsenoside Rf	-O-Glc^2-Glc	-H	-H
Ginsenoside Rg_1	-O-Glc	-H	-Glc

Fig. 10.20 Biosynthetic pathway of phytosterols and triterpenes in *P. ginseng*. Triterpenes undergo oxidation and glycosylation, and are converted into triterpene saponins. Dammarenediol synthase activity was detected in the microsome fraction of *P. ginseng* (Kushiro et al. 1997, 1998)

Table 10.5 Production of metabolites, and their storage in plant systems. Example of compartmentalization in the formation of plant secondary metabolites

Local compartment	Plant species	Secondary metabolite	Synthesis & storage site
Cellular	Asteraceae	Benzofurane Benzopyrane	Specific oil cells
	Catharanthus roseus	Alkaloids	Synthetic capacity depends on the number of storage cells
	Nicotiana rustica	Nicotine	Synthesis in the roots, and storage in the cytosol of leaf cells
	Digitalis lanata	Digitoxin	Mesophyll cells of leaves
	Euphorbia lathyrus	DOPA	Synthesis in leaves, storage in the latex
Subcellular/cell structures	*Coptis japonicum*	Anthraquinones	Rough endoplasmic reticulum (ER)
	Juniperus communis	Tannins	Vacuoles
	Lithospermum erythrorhizon	Naphthoquinones	Naphthoquinone vesicles: rough endoplasmic reticulum
	Papaver somniferum	Alkaloids	Alkaloid vesicles
	Pinus elliotti	Tannins	ER and Golgi vesicles
Subcellular/membrane formation/ multi-enzyme complexes	*Haplopappus gracilis*	Naringenin Eriodictyol	Endoplasmic reticulum
	Sorghum bicolor	Coumaric acid p-hydroxy-mandelicnitrile	Endoplasmic reticulum, microsomes
Subcellular/various precursor pools		Malic acid	Mitochondria, vacuoles

databases has revealed that many transporters and channels exist in the plant genome. Studies of the genetic sequences that encode these proteins, and of phenotypes caused by the mutation of these sequences have been used to characterize the membrane transport of plant secondary metabolites (Yazaki 2005). Such studies have clarified that not only genes that are involved in the biosynthesis of secondary metabolites, but also genes that are involved in their transport would be important for systematic metabolic engineering aimed at increasing the productivity of valuable secondary metabolites in planta (Yazaki 2005).

The mechanism for the long-distance transport of alkaloids is well elucidated in Solanaceae. Nicotine biosynthetic enzymes are expressed specifically in the root tissues, which is advantageous for the xylem transport of nicotine (Hashimoto and Yamada 2003). The transporter that is involved in the translocation of nicotine has not yet been identified, but a multidrug resistance protein (MDR)-like transport activity was recorded in the Malpighian tubules of tobacco hornworm, *Manduca sexta*.

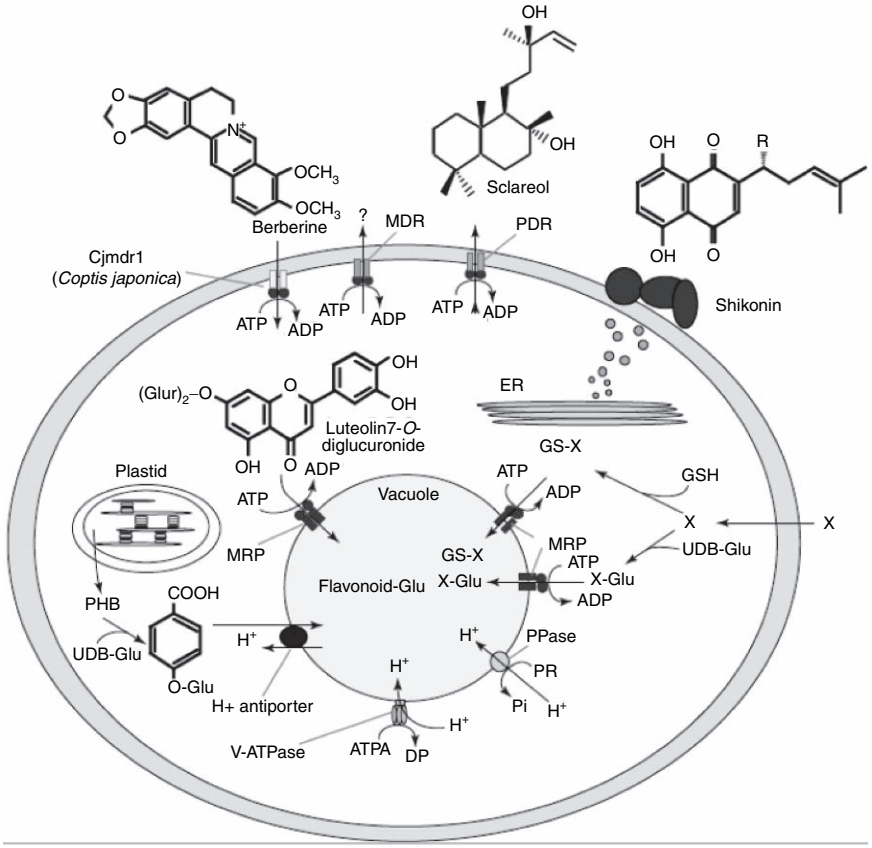


Fig. 10.21 Model of transport processes for secondary metabolites in a plant cell (Yazaki 2005)

Plant alkaloids are often effluxed by ABC transporters in microorganisms and herbivorous insects, but only a few of these transporters are currently known to be responsible for alkaloid transport in planta. Recent studies show that the uptake of an isoquinoline alkaloid, berberine, by *Coptis japonica* cells is mediated by an ABC transporter (Sakai et al. 2002). Functional analyses of CjMDR1 using *Xenopus* oocytes showed that this protein recognized berberine as its substrate, and transported it in an inward direction (Shitan et al. 2003), although most eukaryotic ABC transporters are known to function as efflux carriers. Because berberine is biosynthesized in root tissues, this alkaloid is translocated to the rhizome where it is trapped.

Transport of alkaloids across the tonoplast and other cell membranes has also been shown to involve active mechanisms. Secretion of berberine from cell cultures of *Thalictrum minus* (Yamamoto et al. 1987), and benzophenanthridine alkaloids from *Eschscholzia californica* were both found to be vanadate-sensitive. A more

recent study isolated an ABC transporter involved in the secretion of an antifungal terpenoid alkaloid from a cell culture of *Nicotinia plumbaginifolia* (Jasinski et al. 2001).

Tropane alkaloids are localized in the vascular region where large cells in the secondary xylem are reported to be present. In some alkaloid-producing plant species, alkaloid transport is mediated by carrier proteins. Wink (1985) reported significant alkaloid production when gene expression for alkaloid biosynthesis and transport (which is related to accumulation) took place at the same time. Guern et al. (1987) reported that gene expression for both alkaloid biosynthesis and transport takes place in the presence of a suitable storage site where accumulation without degradation occurs. Since large xylem cells are dead, biosynthesis of enzymes is not possible in these cells. The gene expression for alkaloid biosynthesis and the carrier proteins thus takes place after formation of the large cells in the secondary xylem. Formation of alkaloids in the non-rooted shoots may also be related to other cells. Further investigations related to other cell differentiation are therefore necessary.

When the concentration of a highly toxic secondary metabolite is increased by genetic engineering, does its intrinsic toxicity become a limiting factor? Recent experiments suggest that this might be the case. Nicotine, and also other alkaloids are highly toxic to plant cells, but overexpression of the yeast ABC transporter PDR5 in transgenic tobacco cells was recently demonstrated to decrease the cellular toxicity (Goossens et al. 2003b).

10.9 Bioreactors (see also Sect. 3.2)

Generally, the plant products of commercial interest are secondary metabolites belonging to three main categories, i.e., essential oils, glycosides, and alkaloids. This categorization differs from the one given at the beginning of this chapter. Whereas the earlier one is oriented more on chemical definitions, here the definition is more from a point of view of application. The essential oils consist of a mixture of terpenoids, which are used as flavoring agents, perfumes, and solvents. The glycosides include flavanoids, saponins, phenolics, tannins, cyanogenic glycosides, and mustard oils, which are utilized as dyes, food colors, and medicinals (e.g., steroid hormones, antibiotics). The alkaloids are a diverse group of compounds with over 4,000 structures known. Almost all naturally occurring alkaloids are of plant origin. Alkaloids are physiologically active in humans (e.g., cocaine, nicotine, morphine, strychnine), and therefore of great interest for the pharmaceutical industry (Shuler 1981). However, various problems associated with low cell productivity, slow growth, genetic instability of high-producing cell lines, poor control of cellular differentiation, and inability to maintain photoautotrophic growth have limited the application of plant cell cultures (Sajc et al. 2000).

As described above, in 1983 for the first time a dye, shikonin, with anti-inflammatory and antibacterial properties, was produced by plant cell cultures on

an industrial scale by Mitsui Petrochemical Industries Ltd (Fujita et al. 1982). Although this was thought to be a major breakthrough, shikonin was still up to the 1990s the only plant compound to be produced on a commercial scale by cell cultures.

There are several means of increasing the production of secondary metabolites by plant cell cultures or suspensions, as has been discussed before:

- use of biotic or abiotic elicitors
- addition of a precursor of the desired compound
- secondary metabolite production, or inducing changes in the flux of carbon to favor the expression of pathways leading to the target compound
- production of new genotypes by means of protoplast fusion, or genetic engineering
- use of mutagens to increase the variability already existing in living cells
- use of root cultures.

At the European level, some years ago all these issues were discussed comprehensively at the Symposium on "Primary and Secondary Metabolism of Plant Cell Cultures" (Neumann et al. 1985), and at a seminar on "Bioproduction of Metabolites by Plant Cell Cultures" held in Paris in September 1988, organized by the International Association of Plant Tissue Culture and the French Association pour la Promotion Industrie-Agriculture (APRIA, Association for the Promotion of Industry-Agriculture). However, constrains still remain that need high investments, and finding solutions to the problems of raising plant tissues in bioreactors.

Economic considerations govern the importance attached to the production of natural substances and biochemicals by cell cultures. Some additional information to those given earlier can be obtained from the data in Table 10.6. The estimated annual market value of pharmaceutical products of plant origin in industrialized

Table 10.6 Economic data for some substances of plant origin (t, tons)

Substance and use	Annual demand	Industrial cost (US$ per kg)	Estimated annual market value (in US$ million)
Pharmacy			
Ajmalicine	3–5 t	1,500	4.5–7.5
Codeine	80–150 t	650–900	52–135
Digoxin	6 t	3,000	18
Diosgenin	200 t	20–40	4–8
Vinblastine, vincristine	5–10 kg	5 million	25–50
Food additives, and fragrances			
Jasmine oil	100 kg	5,000	0.5
Mint oil	3,000 t	30	90
Natural vanillin	30 t	2,500	75
Cosmetics			
Shikonin	150 kg	4,000	0.6

countries was over US$ 20 billion in the mid-1980s. The annual market value of codeine, and of the antitumor alkaloids vinblastine and vincristine has been estimated at about US$ 100 million per product (Pétiard and Bariaud-Fontanel 1987). The worldwide market value of aromas and fragrances was expected to rise to US$ 6 billion in 1990 (Rajnchapelmessai 1988).

In 1988, the estimated annual market value of shikonin (for details, see below) was about US$ 600,000, which is far from the US$ 20–50 million investment of the original research and development work. However, the final cost of the product fell to US$ 4,000 per kg, which is similar to US$ 4,500 per kg for the substance extracted from the roots of *Lithospermum erythrorhizon* (Sasson 1991).

It should be noted that Kanebo, the Japanese cosmetics corporation that developed lipsticks containing shikonin, realized a turnover of about US$ 65 million over 2 years in Japan through the sale of 5 million lipsticks, each selling for US$ 13. In the Republic of Korea and in China, Mitsui Petrochemicals Ltd today intends to sell at over US$ 4 billion (as estimated).

In the Federal Republic of Germany, Alfermann et al. (1985), in collaboration with Boehringer Mannheim AG, were able to grow cells of *Digitalis lanata* in 200-l bioreactors, and obtain 500 g of beta-methyldigoxin in 3 months (see also above); the bioconversion rate of beta-methyldigitoxin was very high, up to 93.5%, if the non-used substrate was recycled. Ulbrich et al. (1985) cultured *Coleus blumei* cells in a 42-l bioreactor fitted with the module spiral stirrer, using this system with aeration. They reported high yields of rosmarinic acid (5.5 g/l), representing 21% dry weight of cells. Heble and Chadha (1985a, b) reported the successful cultivation of *Catharanthus roseus* cells in 7- to 20-l capacity bioreactors, modified to provide airlift and agitation, in single and multiple stages.

The cells produced high levels of total alkaloids, comprising ajmalicine and serpentine as the major components. It was shown that plant cells could withstand shear to some extent, and that judicious use of airlift, and low agitation were advantageous. Researchers at Ciba-Geigy AG, Basel, Switzerland, have produced the alkaloid scopolamine from cell cultures of *Hyoscyamus aegypticus* grown in airlift bioreactors.

10.9.1 Technical Aspects of Bioreactor Systems

Bioreactor studies represent the final step that leads to a possible commercial production of secondary metabolites from plant cell cultures. This is an important phase, as numerous problems arise when scaling up the work realized in Erlenmeyer flasks. For example, growth is considerably modified when cells are cultivated in large tanks, and the production of cell biomass remains a critical point for bioreactor productivity. Here, some of the guidelines for upscaling will be given (Figs. 10.22, 10.23, 10.24).

Despite potential advantages in the production of secondary metabolites in plant cell cultures, other than shikonin, only ginsenosides and berberine are today

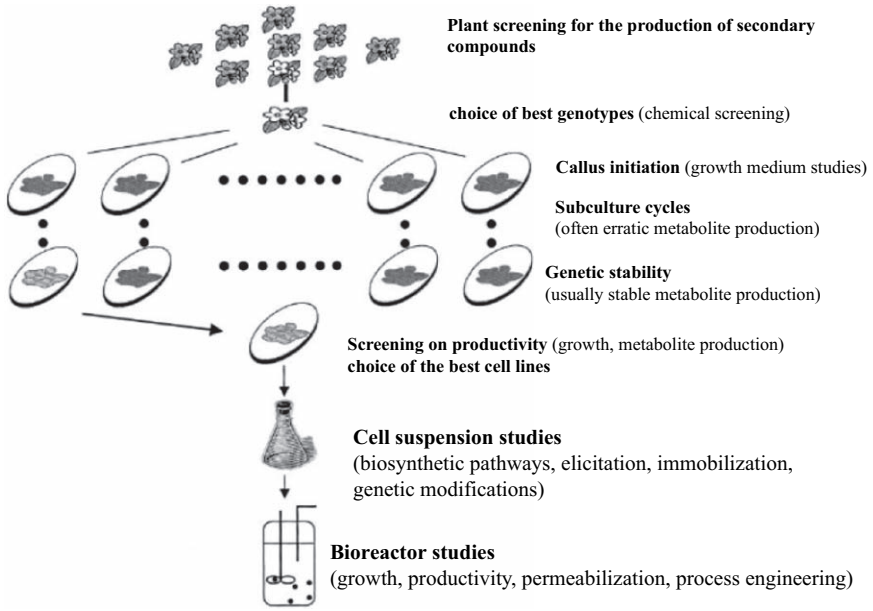


Fig. 10.22 Guidelines for the production of secondary metabolites from plant cells (from Bourgaud et al. 2001)

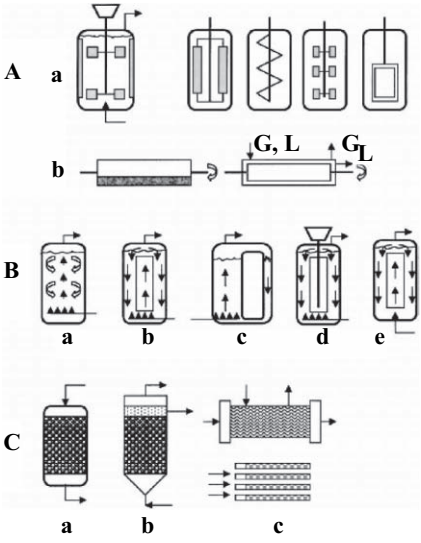


Fig. 10.23 Bioreactor types for plant cell, tissue, and organ cultures. *A* Mechanically agitated bioreactors: **a** stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), **b** rotary drum tank reactor. *C* Air-driven bioreactors: *a* bubble column, *b* concentric tube airlift reactor (IL ALR), **c** external loop airlift

produced on a large scale, and all three processing plants are located in Japan (Hara 1996). The anticancer drug Taxol (registered trademark of Bristol-Myers Squibb) is under consideration for large-scale production (Seki et al. 1995, 1997; Roberts and Shuler 1997).

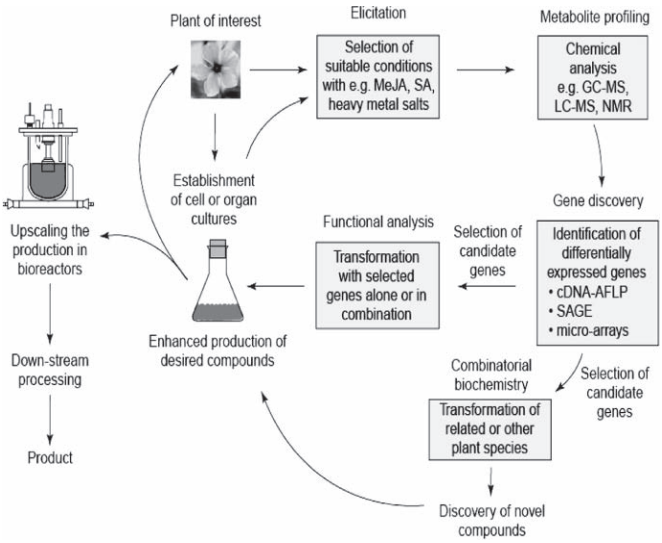


Fig. 10.24 Outline of how functional genomics could contribute to enhancing the production of known and novel secondary metabolites in plant cells. *AFLP* Amplified fragment length polymorphism, *GC-MS* gas chromatography–mass spectrometry, *LC-MS* liquid chromatography–mass spectrometry, *MeJA* methyljasmonate, *NMR* nuclear magnetic resonance, *SA* salicylic acid, *SAGE* serial analysis of gene expression (after Oksman-Caldentey and Inze 2004)

Problems are due mainly to mass transfer limitations of oxygen (Jones and Veliky 1981; Hulst et al. 1985), as well as inhomogeneous culture systems that cause cell sedimentation and death. Recent studies have confirmed the low percentage of viable cells (approx. 50%) generally present in such liquid systems, except for the first days of culture (Steward et al. 1999).

Often, another strong limitation of growth is due to plant cell sensitiveness to shear stress, which is responsible for extensive cell death. This lysis is a consequence of the agitation of the culture medium.

Fundamental studies of bioreactors with plant cells involve three important scientific and practical issues related to bioreactor design and operation: (1) assessment of cell growth and product formation; (2) analysis and modeling of the culture dynamics, including the integration of biosynthesis and product separation; and (3) studies of flow, mixing, and mass transfer between the phases, in order to define criteria for bioreactor design and scale up. For a given application, the culture conditions can be optimized with respect to cell support, medium composition and renewal rate, mass transfer of chemical substances, and bioreactor fluid dynamics, in order to define the conditions that are permissive for, or even designed to promote selected cell functions.

Large-scale suspension culture of ginseng cells was first reported by Yasuda et al. (1972). Later, industrial-scale culturing was initiated by Nitto Denko Corporation (Ibaraki, Osaka, Japan) in the 1980s, using 2,000- and 20,000-l stirred tank fermenters to achieve productivities of 500–700 mg/l per day (Furuya 1988;

Ushiyama 1991). This process is considered an important landmark in the commercialization of plant tissue and cell culture on a large scale.

Prenosil and Pedersen (1983), Payne et al. (1987), Panda et al. (1989), and Scragg (1991) reviewed different reactor configurations for plant cell suspensions, plant tissue, and organ cultures (Fig. 10.23). The relative advantages and selection criteria for various reactor configurations were discussed for specific process applications. In particular, bioreactors that integrate biosynthesis with product release and separation were most extensively studied in Japan (Uozumi et al. 1991; Honda et al. 1993).

Numerous modifications of the conventional stirring tank reactor (STR) with bubble aeration have been developed by employing a variety of impeller designs. The controllability and flexibility of the STR, in terms of independent adjustment of mixing and aeration, makes it the most frequently chosen configuration, despite several limitations such as high power consumption, high shear, and problems with sealing and stability of shafts in tall bioreactors. Although membrane reactors and packed bed reactors (Fig. 10.23) are advantageous, in that a large amount of cells can be immobilized per unit volume (see above), diffusional limitations of mass transfer to the immobilized cells, as well as the difficulties in supplying and removing gaseous components can limit the use of both configurations to biotransformations. Airlift bioreactors (ALR) using low-density beads with immobilized cells or enzymes are currently under research for a variety of applications in bioprocess engineering, and they have several advantages over alternate bioreactor designs. Airlift bioreactors combine high loading of solid particles and good mass transfer, which are inherent for three-phase fluidized beds (Fig. 10.23). Efficient mixing in the liquid phase is generated by air bubbles, using internal (IL ALR) or external (EL ALR) recirculation loops (Fig. 10.23).

A typical recovery process involves four separation steps. The starting feed stream contains particulate material that must be removed by, e.g., centrifugation and/or filtration. Dilute solution of the product is then concentrated using, e.g., nonselective separation techniques (ultrafiltration, precipitation, liquid–liquid extraction, and adsorption). The subsequent steps involve a series of purifications to capture the product, and remove trace contaminants. Chromatography, in its various forms (molecular size, charge, hydrophobicity, and molecular recognition), has proved to be the only general separation technique that can simultaneously achieve high purity, retain biological activity, and be scaled up to an appropriate production capacity.

A great deal of attention has been given to methods of capturing products directly from dilute and particulate-laden feed streams, thereby eliminating the need for all concentration steps. Affinity chromatography is such a technique, and in cases where high specificity and sensitivity are required, antibodies are an ideal choice for the separation of biomolecules, because of their high binding strength and selectivity (Birnbaum and Mosbach 1991). Although extremely versatile, packed bed separations (adsorption, absorption, ion exchange, and gel and affinity chromatography) are limited to batch operation, and are not capable of handling cells or particulate material. Continuous separations can be performed using a magnetically stabilized

fluidized bed (MSFB) of ferromagnetic particles, or a mixture of nonmagnetic and magnetically susceptible support. MSFB has the flow and mass transfer properties of a packed bed, in conjunction with the solid phase fluidity of a fluidized bed. Continuous separations can thus be performed by countercurrent contact of solid and liquid phases without significant longitudinal mixing (i.e., in plug flow) at a low operating pressure drop. Recent applications of MSFB in biotechnology include continuous protein recovery, plant cell filtration, and plant cell cultivation.

The synthesis and excretion of secondary products are often coupled, and associated with membrane transport of the product (see above). An artificial accumulation site can therefore reproduce similar transport phenomena in vitro. Luckner (1980) indicated that the productivity of plant cells can be improved by integration of biosynthesis and product recovery in the extractive phase. Additional advantages of integrated production and separation include enhanced rates of mass transfer, decreased level of product inhibition, facilitated product recovery, and reduced reaction volume for a given amount of product.

10.10 Prospects

With the onset of the 1990s, only Japan, and to a lesser extent, the Federal Republic of Germany were really engaged in the industrial production of secondary metabolites by plant cell cultures.

The only marketed product (as of 1990) remains shikonin. In Japan, seven private corporations have created a common subsidiary in research and development on plant cell cultures. Plant Cell Culture Technology (PCC Technology) has been set up with the support of the Japan Key Technology Centre (JKTC) by Kyowa Hakko Kogyo Co., Mitsui Petrochemical Industries Ltd, Mitsui Toatsu Chemical Inc., Hitachi Ltd, Suntory Ltd, Toa enryo Kogyo Co., and Kirin Breweries Co. Ltd. By contrast, most North American and European companies have not been enthusiastic about the prospects of profitable industrial production (Rajnchapelmessai 1988).