Adv Biochem Engin/Biotechnol (2005) 100: 53–88
DOI 10.1007/b136412

Published online: 5 July 2005

Plant Cells: Secondary Metabolite Heterogeneity and Its Manipulation

Jian-Jiang Zhong[1] (✉) · Cai-Jun Yue[1,2]

[1]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 200237 Shanghai, P.R. China
jjzhong@ecust.edu.cn

[2]College of Life Science and Biotechnology, Heilongjiang August First Land Reclamation University, 163319 Daqing, P.R. China

Abstract This chapter proposes the concept of rational manipulation of secondary metabolite heterogeneity in plant cell cultures. The heterogeneity of plant secondary metabolites is a very interesting and important issue because these structure-similar natural products have different biological activities. Both taxoids and ginsenosides are two kinds of preeminent examples in the enormous reservoir of pharmacologically valuable heterogeneous molecules in the plant kingdom. They are derived from the five-carbon precursor isopentenyl diphosphate, produced via the mevalonate or the non-mevalonate pathway. The diterpenoid backbone of taxoids is synthesized by taxadiene synthase and the triterpenoid backbone of ginsenosides is synthesized by dammarenediol synthase or β-amyrin synthase. After various chemical decorations (oxidation, substitution, acylation, glycosylation, benzoylation, and so on) mediated by P450-dependent monooxygenases, glycosyltransferases, acyltransferases, benzoyltransferases, and other enzymes, the terpenoid backbones are converted into heterogeneous taxoids and ginsenosides with different bioactivities. Although detailed information about accumulation and regulation of individual taxoids or ginsenosides in plant cells is still lacking, remarkable progress has recently been made in the structure and bioactivity identification, biosynthetic pathway, manipulation of their heterogeneity by various methodologies including environmental factors, biotransformation, and metabolic engineering in cell/tissue cultures or in plants. Perspectives on a more rational and efficient process to manipulate production of de-

sired plant secondary metabolites by means of metabolic engineering and "omics"-based approaches (e.g., functional genomics) are also discussed.

Keywords Plant cell · Heterogeneity · *Taxus* spp. · Ginseng · Manipulation · Secondary metabolite

1 Introduction

Higher plants, about 400 000 species in the world [1], are a valuable source of numerous metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. More than 100 000 plant secondary metabolites have already been identified, which probably represent only 10% of the actual total in nature and only half the structures have been fully elucidated [2–4]. Molecular diversity is a widely existing phenomenon in nature, and many plant secondary metabolites are structure-similar but bioactivity-different. The enormous heterogeneity of plant secondary metabolites is usually derived from differential modification of common backbone structures. For example, over 5000 different flavonoids and 300 different glycosides of a single flavonol, quercetin, have already been identified [5]. The immense diversity of plant secondary metabolites is often obtained by derivatization of specific lead structures through post-biosynthetic events such as hydroxylation, glycosylation, methylation, acylation, prenylation, sulfation, and benzoylation [6]. Hundreds of secondary metabolite modifying enzymes (e.g., oxidases, acyltransferases, methyltransferases, glycosyltransferases, sulfotransferases, and benzoyltransferase) have been cloned and characterized [7, 8].

Generally, the function of each plant secondary metabolite is different. Figure 1 shows terpenoids as an extremely fascinating example; they are present in all organisms but are especially abundant in plants, with more than 30 000 compounds reported to date [9–11]. Terpenoids are the most functionally and structurally diverse group of plant natural products that include diterpenoid alkaloids, sterols, triterpene saponins, and related structures. The most basic function of triterpenes is to give membranes stability, such as β-sitosterol (**1** in Fig. 1) does in plants. By further oxygenation, for example, castasterone (**2** in Fig. 1), acts as signals that interfere with morphological differentiation in plants. Furthermore, triterpene glycosides, such as saponin phytoalexins (**3** in Fig. 1), damage fungal membranes by significantly reducing their stability [12].

Many structure-similar but bioactivity-different secondary metabolites are usually generated in one plant. Both taxoids (diterpenoid alkaloids originally isolated from the bark of the Pacific yew, *Taxus brevifolia*) and ginseng saponins (ginsenoside, an active group of triterpene saponins mostly from

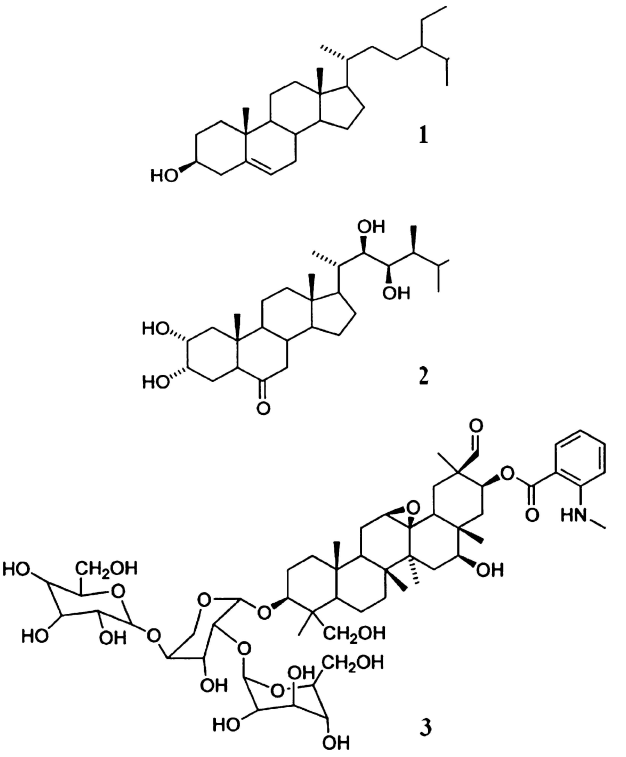


Fig. 1 Triterpenes with diverse biological activities: β-sitosterol (**1**) confers membrane stability in plants; castasterone (**2**), a brassinosteroid growth hormone; avenacin A-1 (**3**), antifungal saponin phytoalexin. Refer to the text for details

Panax ginseng, *P. notoginseng* or *P. quinquefolium*) are tremendously heterogeneous. Anticancer potency of each taxoid is different [13]. The biological activities of some ginsenosides even oppose each other. For example, Rg_1 has the effect of stimulating the central nervous system, whereas Rb_1 has tranquilizing effects on the central nervous system and Rc inhibits the central nervous system [14, 15]. However it is difficult to manipulate their heterogeneity in field-cultivated plants; therefore, the pharmacodynamic instability of these herbs often takes place owing to the change of the quality of the raw materials (especially in both the composition and the distribution of related metabolites). The purification of an individual compound is a current approach for maintaining certain specific potency, but the metabolite (taxoid and ginsenoside) content is usually quite low, while the physicochemical characteristics of various analogues (taxoids or ginsenosides) are very similar; therefore, their separation and purification is an expensive and very complicated process, and the yields of active compounds from plants are season- and environment-dependent. Cell and tissue culture is an attractive alterna-

tive source to a whole plant for production of the high-value-added secondary metabolites. This chapter proposes the concept of rational manipulation of secondary metabolite heterogeneity in plant cell cultures. It is very advantageous to intentionally manipulate the heterogeneity of secondary metabolites in plant cell and tissue cultures by altering or stimulating their genome and/or the subsequent processes, which result in the desired enzymatic syntheses of secondary metabolites. The manipulating techniques utilized include elicitation, hormone treatment, enzyme inhibition, growth-retardant treatment, and precursor-directed biosynthesis resulting in the production of previously undiscovered plant metabolites or a change of the production ratio of certain secondary metabolites [16]. Of course, other engineering strategies, such as temperature shift and change of oxygen partial pressure, also affect the heterogeneity of plant secondary metabolites in cell cultures. Biotransformation by various organisms and enzymes is an effective method for changing the heterogeneity of plant secondary metabolites. Metabolic engineering approaches are promising in manipulating the accumulation of plant secondary metabolites. In the following, by taking taxoid and ginsenoside as typical examples, progress in the structure and activity identification, biosynthesis, and manipulation of their heterogeneity in plants, their tissues or cells is reviewed.

2
Heterogeneity of Taxoid and Its Manipulation

2.1
Taxoid and Its Diversity

Taxoids are complex, substituted diterpenoids, one of which, the famous taxol (paclitaxel), was first isolated from the bark of *T. brevifolia* Nutt and its structure was defined in 1971 [17]. Subsequently, paclitaxel and taxoid derivatives have been reported from foliage and bark of several other species of *Taxus*, like *T. wallichinan*, *T. baccata*, *T. canadensis*, *T. cuspidata*, and *T. yunnanesis* [18–22]. In addition to the plant source, some endophytic fungi, such as *Tubercularia* sp., *Sporormia minima*, and *Seimatoantlerium tepuiense*, have also been reported to produce taxol and other taxoids [23–25].

Until now, over 350 taxoids have been classified into 16 groups (Table 1) [26]. Chemical derivatization of taxoids contributes to the diversity of taxoid function. Taxoids are well-known antineoplastic drugs, and are used to treat a range of cancers, either alone or in combination with other chemotherapeutic agents [27, 28]. Guéritte [29] summarized the general structure-antitubulin activity relationship (Fig. 2). Paclitaxel is a highly functionalized taxoid that acts by promoting tubulin polymerization, ultimately leading to

Table 1 Classification of taxoids

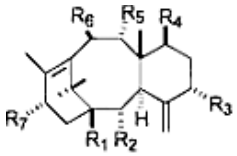
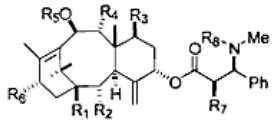
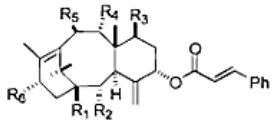
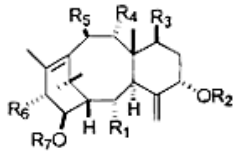
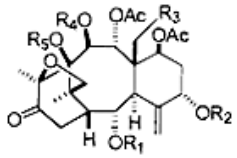
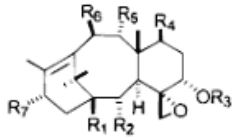
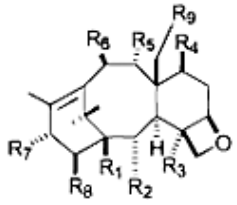
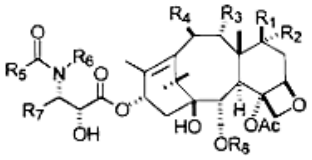
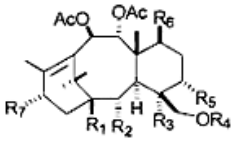
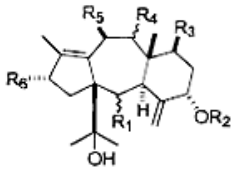
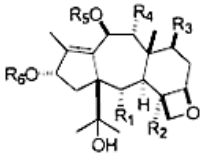
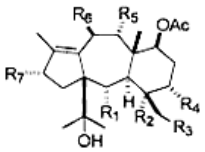
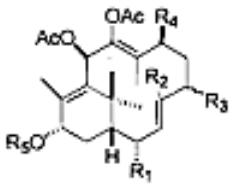
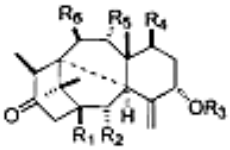
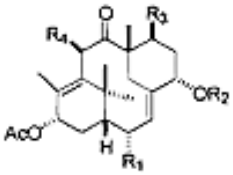
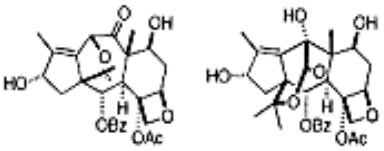
Class	Structure
Neutral taxoids with a C-4(20) double bond	
Basic taxoids with a C-4(20) double bond	
5-Cinnamoyl taxoids with a C-4(20) double bond	
Taxoids with a C-4(20) double bond and oxygenation at C-14	
Taxoids with a C-12(16)-oxido bridge and a C-4(20)double bond	
Taxoids with a C-4(20) epoxide	
Taxoids with an oxetane ring	

Table 1 (continued)

Class	Structure
Taxoids with an oxetane ring and a phenylisoserine C-13 side chain	
Taxoids with an open oxetane or oxirane ring	
11(15*f*1)-*abeo*-Taxoids with a C-4(20) double bond	
11(15*f*1)-*abeo*-Taxoids with an oxetane ring	
11(15*f*1)-*abeo*-Taxoids with an open oxetane or oxirane ring	
3,8-*seco*-Taxoids	

cell death [30]. The structural elements (pharmacophores) responsible for the cytotoxicity of paclitaxel, in addition to the rigid taxane skeleton, include the oxetane ring (D-ring), the *N*-benzoylphenylisoserine side chain appended to C-13, the benzoate group at C-2, and the acetate function at C-4 of the taxane ring [31]. In 120 taxoids isolated from the Japanese yew, *T. cuspidate*, only four non-paclitaxel-type taxoids (taxuspine D, taxezopidines K and L, and

Table 1 (continued)

Class	Structure
Taxoids with a C-3(11) bridge and a C-4(20) double bond	
2(3*f*20)-*abeo*-Taxanes	
Other miscellaneous taxoids	

taxagifine) exhibit potent inhibitory activity against Ca^{2+}-induced depolymerization of microtubules, while taxuspine D induces spindles with strong birefringence in the same manner as paclitaxel [32].

2.2 Taxoid Biosynthesis and Manipulation of Taxoid Heterogeneity

2.2.1 Taxoid Biosynthesis

A typical biosynthetic pathway of taxoids, by taking paclitaxel as an example, is illustrated in Fig. 3. The diterpenoid skeleton of taxoids, as with other terpenoids of plastid origin, was observed by using labeling studies with ^{13}C-labeled glucose to be derived via the 1-deoxy-D-xylulose-5-phosphate pathway [33–37], in which the isopentenyl diphosphate formed is employed in the biosynthesis of carotenoids, phytol, plastoquinone, isoprene, monoterpenes, and diterpenes. The committed step in the biosynthesis of paclitaxel and other taxoids is represented by the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) to taxa-4(5),11(12)-diene [38]. Taxadiene synthase, a 79-kDa diterpene cyclase, catalyzes this reaction, which is slow but apparently not rate-limiting [39, 40].

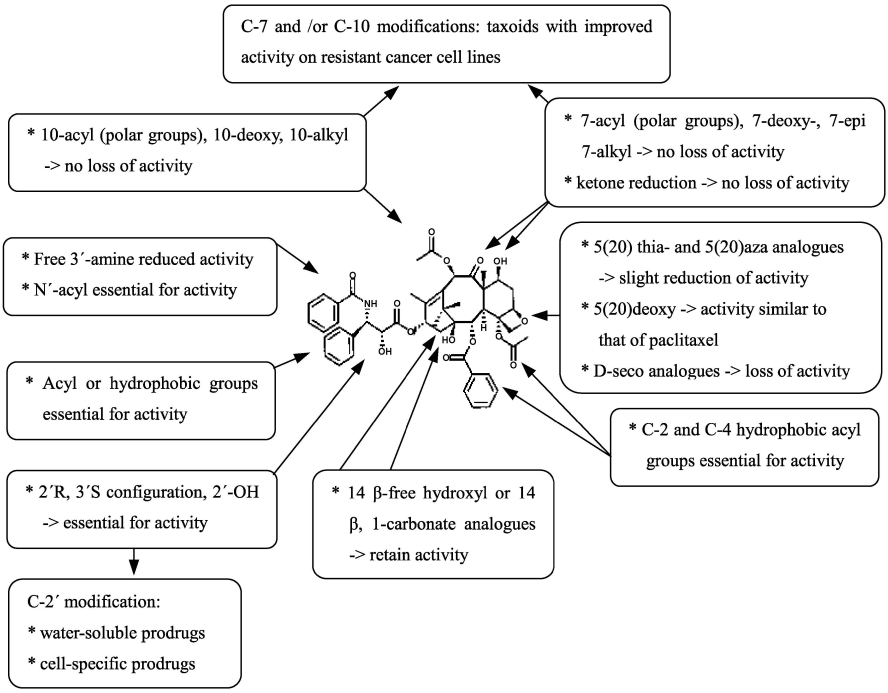


Fig. 2 The general structure–antitubulin activity relationships of taxoids (modified from the literature [29])

On the other hand, the enzyme was demonstrated to be a key one in the biosynthesis of a taxoid, taxuyunnanine C (2α,5α,10β,14β-tetraacetoxy-4(20),11-taxadiene, Tc), by suspended cells of *T. chinensis* in response to methyl jasmonate (MJA) elicitation [41]. The second specific step in taxoid biosynthesis is considered to be the cytochrome P450 dependent hydroxylation at the C-5 position of the taxane ring, which is accomplished by allylic rearrangement of the 4(5) double bond to the 4(20) position to yield taxa-4(20),11(12)-diene-5α-ol [42]. Taxa-4(20),11(12)-diene-5α-ol is a branching point in the paclitaxel pathway to form other naturally occurring taxanes. The enzymes taxadien 13α-hydroxylase and taxadien-5α-ol acetyltransferase, which catalyze taxa-4(20),11(12)-diene-5α-ol to produce different taxoids, were reported [43, 44]. Taxadiene-5α-10β-diol monoacetate was another possible branching point in the paclitaxel pathway. It can be transformed into 5α-acetoxy-10β,14β-dihydroxy taxadiene by taxoid 14β-hydroxylase, but it is still not known how it is transformed into 2-debenzoyltaxane or taxasin [45, 46]. However, previous evaluations [47] of the relative abundance of naturally occurring taxanes [26, 48] have suggested that hydroxylations at positions C-5, C-10, C-9, and C-2 are earlier than that at positions C-13, C-1, and C-7 of the taxane ring in pacli-

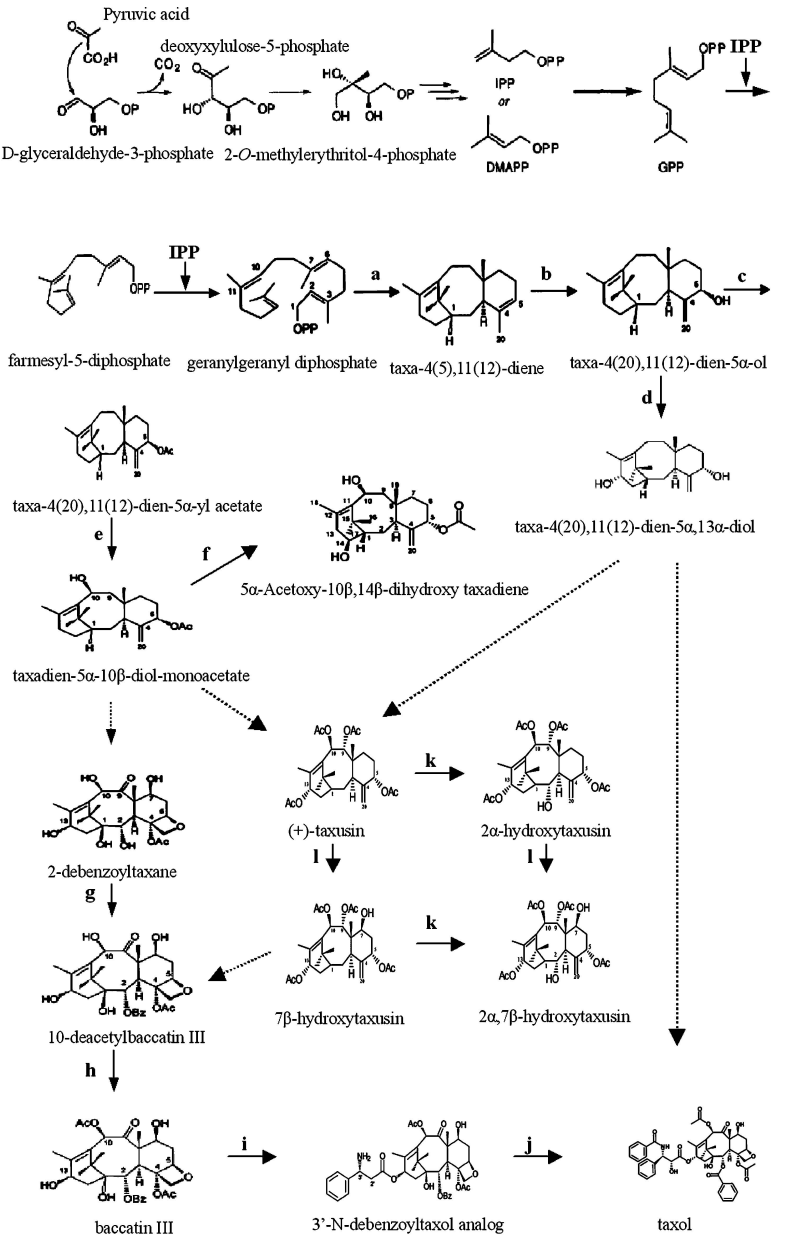


Fig. 3 The proposed paclitaxel biosynthetic pathway. The enzymes indicated are *a* taxadiene synthase, *b* taxadiene 5α-hydroxylase, *c* taxadien-5α-ol acetyltransferase, *d* taxadien 13α-hydroxylase, *e* 10α-hydroxylase, *f* 14β-hydroxylase, *g* 2α-*O*-benzoyltransferase, *h* 10-*O*-acetyltransferase, *i* phenylpropanoyltransferase, *j* 3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase, *k* 7β-hydroxylase, and *l* 2α-hydroxylase. The *broken arrow* indicates multiple convergent steps (modified from Refs. [43–46, 51–54])

taxel biosynthesis, and several biosynthetic mechanisms have been proposed for formation of the oxetane ring (D-ring) [49, 50]. Taxusin, a presumed dead-end metabolite of yew heartwood, may also be from taxa-4(20),11(12)-dien5α,13β-diol and/or taxadiene-5α-10β-diol monoacetate, although the details are unclear. Taxusin is another node in the biosynthesis of taxoids, and can efficiently be converted to the corresponding 2α–hydroxytaxusin and 7β-hydroxytaxusin by the taxoid 2α-hydroxylase and the taxoid 7β-hydroxylase, respectively. It is also possible that 7β-hydroxytaxusin will be converted to 2-debenzoyltaxane [46]. Until now the pathway from 2-debenzoyltaxane to paclitaxel has been clear, and includes the formation of 2-benzoxy taxoid by taxane 2α-*O*-benzoyltransferase, the conversion of 10-deacetylbaccatin III to baccatin III by 10-*O*-acetyltransferase, side-chain attachment by the phenylpropanoyltransferase, and side-chain benzamidation by 3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase to form paclitaxel [51]. Given the very large number of structurally defined taxoids, and that there are even multiple pathways from taxadiene to paclitaxel, there must also exist several side routes and diversions responsible for the formation of various taxoids. The substrate selectivities of the taxoid hydroxylases and acyltransferases almost certainly play a central role in the formation of heterogeneous taxoids.

2.2.2
Manipulation of Taxoid Heterogeneity

Since paclitaxel has been found to exhibit significant antitumor activity against various cancers, and there is poor availability of paclitaxel from natural sources (only 50–150 mg/kg of dried trunk bark can be isolated from several species of yew), great attention has been paid to other supply sources. Except for semisynthesis from its natural precursor 10-deacetylbaccatin III, which is mainly obtained from leaves of *Taxus* species, plant cell and tissue culture of *Taxus* species is considered as one of the most promising approaches to obtain paclitaxel and related taxoids. It is practical to manipulate taxoid heterogeneity in cell cultures via environmental factors and molecular biology techniques.

2.2.2.1
Effect of Temperature Shift

Biosynthesis of taxoids in cultured *Taxus* cells was affected by temperature shift during cultivation. When the temperature was shifted from 24 to 29 °C at day 21 in cell cultures of *T. chinensis* treated with 4 μM silver nitrate at the initial cultivation time, the yield of paclitaxel increased from 49.6 to 82.4 mg/L at day 35, while that of Tc decreased from 885.9 to 512.9 mg/L [55]. The results imply that the biosyntheses of different taxoids might have their own

temperature preference, and the temperature-shifting strategy to produce a specific taxoid by cultured cells should be varied accordingly.

2.2.2.2 Effect of Methyl Jasmonate

New taxoids may be produced or primary taxoids lost in cultured *Taxus* cells after elicitation with MJA, a key signal compound which is widely used in the production of secondary metabolites by plant cells. In the CR-5 callus culture of *T. cuspidate* [56], it is reported that after stimulation with 100 μM MJA, five more taxoids, cephalomannine, 1β-dehydroxybaccatin VI, taxinine NN-11, baccatin I, and 2α-acetoxytaxusin, and one more abietane, taxamairin C, were produced in addition to known taxoids, paclitaxel, 7-*epi*-taxol, taxol C, baccatin VI, taxayuntin C, taxuyunnanine C and its analogues, and yunnanxane, and an abietane, taxamairin A. After 60-days elicited cultivation, the levels of taxuyunnanine C and its analogues increased 3.1-fold, and paclitaxel and its analogues increased 5.2-fold compared with those in CR-5 without MJA elicitation. The production of phenolic abietane derivatives, taxamairin A and taxamairin C, was promoted a little [56]. Ketchum et al. [57] reported that after MJA elicitation Mh00D cell lines of *T.* x *media* cv. *Hicksii* produced a new taxoid, 1β-dehydroxybaccatin VI, and lost baccatin III and 10-deacetylbaccatin III, but Mh00W cell lines of *T.* x *media* cv. *Hicksii* produced new taxoids, 1β-dehydroxybaccatin VI, baccatin III, and 5α,7β,9α,10β,13α-pentaacetoxy-2*a*-benzoyloxytaxa-4(20),11-diene, and lost baccatin VI. These results imply that MJA altered the heterogeneity of taxoids by activating certain pathways of taxoid synthesis and/or reducing certain primary pathways in different cell lines. It is necessary to have the metabolic and physiological characterization of cell lines while manipulating the heterogeneity of the products.

In *T. canadensis* (CO93P) suspension cultures with or without 200 mM MJA elicitation, the distribution of taxoids was similar [58]. All of the major taxoids present in the elicited cultures were also present in the nonelicited cultures, but the relative proportion of the taxoids was different. These observations may indicate that MJA elicitation affects the relative abundance of existing taxoids in certain *Taxus* species, even if elicitation does not result in the production of novel taxoids. This may be caused by the accumulation of intermediates as a result of one or more rate-limiting steps in the taxoid biosynthetic pathway.

2.2.2.3 Effect of Precursors, Growth Retardants, and Phenylalanine Ammonia Lyase Inhibitors

Veeresharm et al. [59] reported that precursors and growth retardants showed different improvement of the production of paclitaxel, deacetylbaccatin III, and baccatin III in *T. wallichiana* cell cultures (Fig. 4). The accumulation of deacetylbaccatin III, baccatin III, or paclitaxel enhanced by addition of the precursors phenylalanine (1 mM), sodium benzoate (0.2 mM), hippuric acid (1 mM), and leucine (1 mM) was different in cell cultures. Hippuric

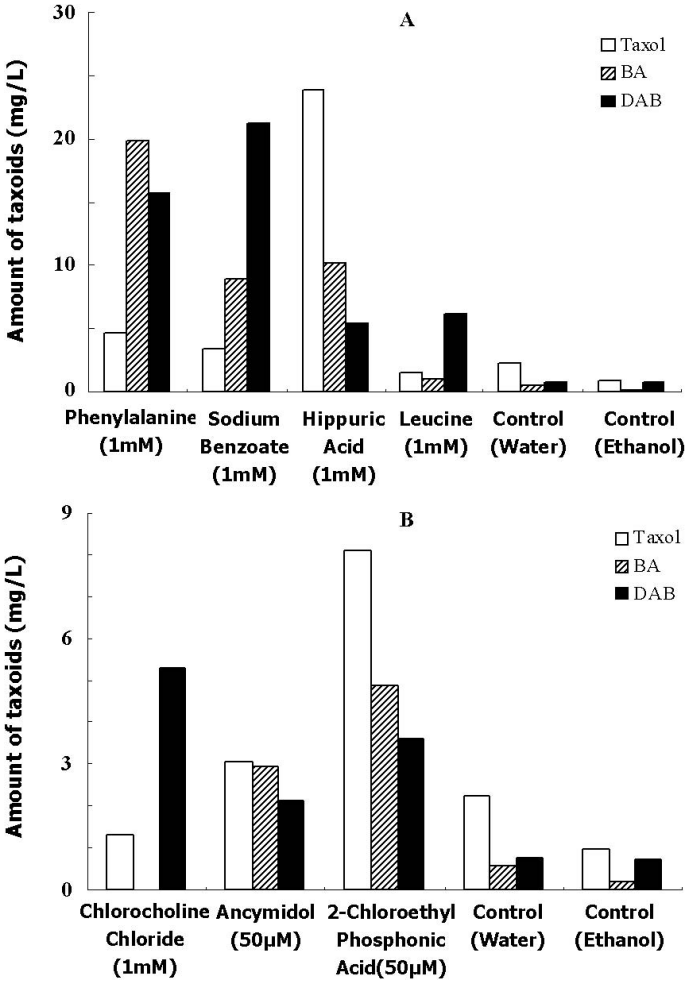


Fig. 4 Effect of **a** precursors and **b** growth retardants on taxoid production in cell cultures of *Taxus wallichiana* (modified from Ref. [56])

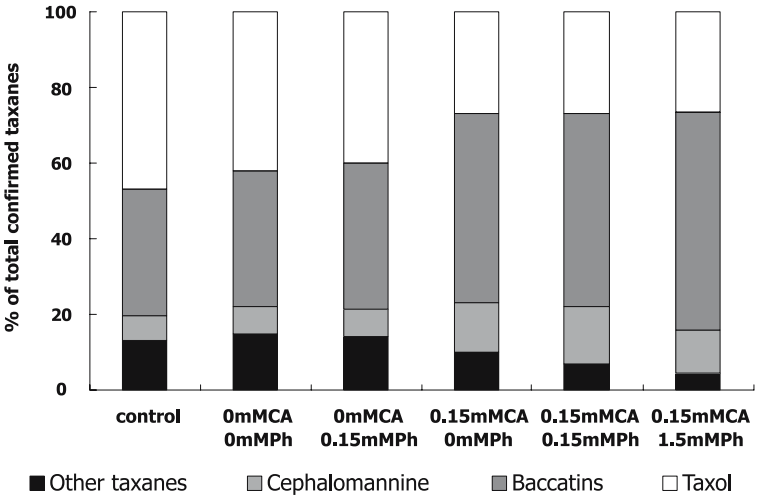


Fig. 5 Single or combined addition of cinnamic acid (*CA*, 0.15 mM) and phenylalanine (*Ph*, 0.15 and 1.5 mM) to CO93P *T. canadensis* cultures at day 7. Taxoids were measured at day 15. The baccatins consist of greater than 96% 13-acetyl-9-dihydrobaccatin III and 9-dihydrobaccatin III (modified from Ref. [57])

acid was most favorable for accumulation of paclitaxel, sodium benzoate for baccatin III, and phenylalanine for deacetylbaccatin III. Like precursors, growth retardants 2-chloroethyl phosphonic acid (50 μM) and chlorocholine chloride (1 mM) were beneficial to the production of paclitaxel and deacetylbaccatin III, respectively. This may be due to the different response of 2α-*O*-benzoyltransferase, 10-*O*-acetyltransferase, phenylpropanoyltransferase, and 3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase to these precursors and growth retardants. These precursors and growth retardants can be potential regulators of the taxoid heterogeneity.

Brincat et al. [60] reported the effect of cinnamic acid (a phenylalanine ammonia lyase, PAL, inhibitor) and phenylalanine on the synthesis of total taxanes in CO93P *T. canadensis* cultures (Fig. 5). The concentration of 13-acetyl-9-dihydrobaccatin III and 9-dihydrobaccatin III at least doubled in CO93P cells treated with 0.15 mM cinnamic acid, although phenylalanine had very little effect on the taxane profile. Considering α-aminooxyacetic acid (a PAL inhibitor), which almost entirely shut down paclitaxel production, and L-α-aminooxy-β-phenylpropionic acid (another PAL inhibitor), which slightly enhanced paclitaxel production, they suggested that the impact of cinnamic acid on paclitaxel might be related not to its effect on PAL but rather to a specific effect on the taxane pathway.

2.2.2.4 Biotransformation

Biotransformation is a biosynthetic or degradation process using enzymes in living organisms or isolated from living cells as biocatalysts. The characteristics of biotransformation are regioselective and stereoselective reaction under mild conditions and easy production of optically active compounds. It is one of the methodologies to produce diverse taxoids. The investigation of biotransformation of taxoids is gaining more and more interest, with their reactions performed by bacteria, fungi, plant cells, and isolated enzymes. Hydroxylation, acylation, epoxidation, hydrolysis, recomposition, and other reactions are generated in biotransformation of taxoids. For example, sinenxan A (a taxoid) can be easily transformed by many organisms (Fig. 6, Table 2). Taxoids can also be transformed directly by various cell-free enzymes, which are very useful in manipulation of taxoid heterogeneity. Patel [68] reported that C-13 taxolase (which catalyzes the cleavage of the C-13 side chain of various taxanes) derived from *Nocardioides albus* SC 13911, C-10 deacetylase (which catalyzes the cleavage of C-10 acetate of various taxanes) derived from *N. luteus* SC 13912, and C-7 xylosidase (which catalyzes the cleavage of C-7 xylose from various xylosyltaxanes) derived from *Morexella* sp. SC 13963 converted various taxanes in extracts of *Taxus* cultivars to 10-deacetylbaccatin III, whose concentration was increased by 5.5- to 24-fold. The C-10 deacetylase also can transform 10-deacetylbaccatin III to baccatin III with a reaction yield of 51% [69]. Recently, conversion from 7-deoxy-10-deacetylbaccatin III into 6-hydroxy-7-deoxy-10-deacetylbaccatin III by *N. luteus* SC 13912 (ATCC 55426) was reported [70].

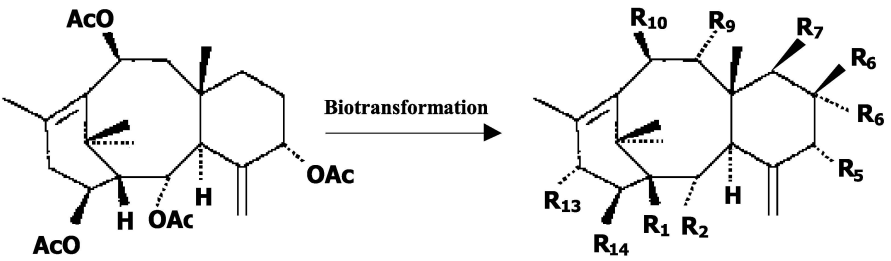


Fig. 6 Biotransformation of sinenxan A by various organisms. The *R* groups and biocatalysts are shown in Table 2

Table 2 Biotransformation of sinenxan A by various organisms

Structures of products	Species of organisms
$R_5 = OH, R_2 = R_{10} = R_{14} = AcO$	*Catharanthus roseus* [61]
$R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*Platycodon grandiflorum* [61, 62]
$R_1 = OH, R_5 = R_9 = R_{10} = R_{13} = AcO$	*Absidia coerulea* [63]
$R_{14} = OH, R_5 = R_9 = R_{10} = R_{13} = AcO$	*A. coerulea* [63]
$R_5 = R_{10} = R_{14} = OH, R_2 = AcO$	*Cunninghamella echinulata* [64]
$R_5 = R_6 = R_{10} = R_{14} = OH, R_2 = AcO$	*C. elegans* [64]
$R_5 = R_6 = R_{10} = OH, R_2 = R_{14} = AcO$	*C. echinulata* [64]
$R_5 = R_{10} = OH, R_2 = R_6 = R_{14} = AcO$	*C. echinulata* [64]
$R_6 = R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*C. roseus, C. echinulata, Ginkgo biloba* [61, 65, 66]
$R_6 = R_9 = R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*C. roseus, G. biloba* [61, 66]
$R_6 = OH, R_2 = R_5 = R_{10} = R_{14} = AcO$	*C. elegans* [64]
$R'_6 = R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*C. echinulata* [67]
$R_7 = OH, R_2 = R_5 = R_{10} = R_{14} = AcO$	*A. coerulea* [63]
$R_9 = R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*G. biloba* [66]
$R_9 = R_{14} = OH, R_2 = R_5 = R_{10} = AcO$	*G. biloba* [66]
$R_9 = OH, R_2 = R_5 = R_{10} = R_{14} = AcO$	*G. biloba* [66]
$R_9 = OCHO, R_2 = R_5 = R_{10} = R_{14} = AcO$	*G. biloba* [66]
$R_{10} = OCHO, R_2 = R_5 = R_{10} = R_{14} = AcO$	*G. biloba* [66]
$R_6 = R_9 = R_{10} = OH, R_2 = R_5 = R_{14} = AcO$	*C. roseus, G. biloba* [61, 66]

The skeletons of sinenxan A analogs are shown in Fig. 6.

2.2.2.5 Metabolic Engineering Approach

A metabolic engineering approach to engineer cells is a new method for directed production of desired taxoids. It was reported that in *Escherichia coli* cells transformed to express three genes encoding four enzymes of the terpene biosynthetic pathway (including the committed GGPP synthase and taxadiene synthase), taxadiene could be conveniently synthesized in vivo at the unoptimized yield of 1.3 mg/L [71]. Considering a limited pool of precursors to GGPP and the requirement of P450 monooxygenases for further biosynthesis of other taxoids, engineered *E. coli* cells are not better than engineered plant cells; thus, Besumbes et al. [72] reproduced some functional steps of the paclitaxel biosynthetic pathway in *Arabidopsis thaliana* plants to produce taxadiene. A complementary DNA (cDNA) encoding the full-length taxadiene synthase from *T. baccata* was successfully integrated into the *A. thaliana* genome. The constitutive production of the enzyme in *A. thaliana*

led to the accumulation of taxadien, and induction of transgene expression using a glucocorticoid-mediated system consistently resulted in a more efficient recruitment of GGPP for the production of taxadiene, which reached a level 30-fold higher than that (around 20 ng/g dry weight) in plants constitutively expressing the transgene.

3
Heterogeneity of Ginsenoside and Its Manipulation

3.1
Ginsenoside and Its Diversity

Ginsenosides are a group of triterpenoid saponins. More than 30 ginsenosides have been isolated from ginseng plants and their chemical structures have been identified. As shown in Table 3, representative ginsenosides exhibit considerable structural variation. In the same type ginsenosides, they differ from one another by the types of sugar moieties, their number, and their site of attachment. Some sugar moieties present are glucose, xylose, rhamnose, and arabinose. They are usually attached to C-3, C-6, or C-20 with formation of chains of a single sugar moiety or oligosaccharide. Ginsenosides also differ in the number and the site of attachment of hydroxyl groups. Compared with that of protopanaxadiol-type ginsenosides, the aglycone of protopanaxatriol-type ginsenosides (protopanaxatriol) has one more hydroxyl group at C-6, which possibly stems from protopanaxadiol by oxidation. Another factor that contributes to structural differences between ginsenosides is the stereochemistry at C-20. Most ginsenosides that have been isolated are naturally present as enantiomeric mixtures [73, 74]. The binding site of the sugar, the number of hydroxyl groups, and the stereoisomerism of ginsenosides have been shown to influence their biological activities.

Numerous reports have been published on the pharmacological and biological activities of various ginsenosides as summarized in Table 4 [75]. There is a very close relationship between the structure and the function of ginsenosides. Both ginsenoside Rd and Rb_1 are protopanaxadiol-type ginsenosides, which differ only by the presence of two glucose moieties at C-20 in Rb_1 and one glucose moiety in Rd. Except for vasodilating action, they do not share the same pharmacological functions (Table 4). Ginsenosides Rh_1 and Rh_2 are also structurally similar. Rh_2 inhibited in vitro proliferation of lung cancer cells 3LL (mice), Morris liver cancer cells (rats), B-16 melanoma cells (mice), and HeLa cells (human) and stimulated melanogenesis and cell-to-cell adhesiveness, but Rh_1 had no effects on cell growth and cell-to-cell adhesiveness despite its stimulation of melanogenesis [76]. Furthermore, only Rh_2 was incorporated in the lipid fraction of the B16–BL6

Table 3 Representative ginsenosides of ginseng congeners

Ginsenoside	R_1	R_2
Protopanaxadiol type		
Rh_2	Glc	H
F_2	Glc	Glc
Rg_3	Glc(2-1)Glc	H
Rd	Glc(2-1)Glc	Glc
Rb_1	Glc(2-1)Glc	Glc(6-1)Glc
Rb_2	Glc(2-1)Glc	Glc(6-1)Arap
Rb_3	Glc(2-1)Glc	Glc(6-1)Xyl
Rc	Glc(2-1)Glc	Glc(6-1)Araf
Ra	Glc(6-1)Glc(6-1)Glc	Glc(3-1)Glc3-1)Glc
Ra_1	Glc(2-1)Glc	Glc(6-1)Arap(4-1)Xyl
Ra_2	Glc(2-1)Glc	Glc(6-1)Arap(2-1)Xyl
Ra_3	Glc(2-1)Glc	Glc(6-1)Arap(3-1)Xyl
Rs_1	Glc(2-1)Glc(6)Ac	Glc(6-1)Arap
Rs_2	Glc(2-1)Glc(6)Ac	Glc(6-1)Araf
Protopanaxatriol type		
Re	Glc(2-1)Rha	Glc
Rf	Glc(2-1)Glc	H
Rg_1	Glc	Glc
Rg_2	Glc(2-1)Rha	H
Rh_1	Glc	H
F_1	H	Glc
F_3	H	Glc(6-1)Arap
Oleanane type		
Ro	Glc(2-1)Glc	Glc

The skeletons of ginsenosides are shown in Fig. 7.
Glc β-D-glucopyranose, *Arap* α-L-arabopyranose, *Araf* α-L-arabofuranose, *Xyl* β-D-xylopyranose, *Rha* α-*l*-rhamnopyranose, *Ac* acetyl

melanoma cell membrane. Differences in the number of hydroxyl groups have also been shown to influence pharmacological activity. Ginsenosides Rh_2 and Rh_3, which possibly stem form protopanaxadiol, are different only by the presence of a hydroxyl group at C-20 in Rh_2. Both Rh_2 and Rh_3 induced the differentiation of promyelocytic leukemia HL-60 cells into morphological and functional granulocytes, but the potency of Rh_2 was higher [77].

Since the modules with which stereoisomers react in biological systems are also optically active, they are considered to be functionally different chemical compounds [78]. Consequently, they often differ considerably in potency, pharmacological activity, and pharmacokinetic profile. Both 20(*S*) and 20(*R*) ginsenoside Rg_2 inhibited acetylcholine-evoked secretion of catecholamines from cultured bovine adrenal chromaffin cells [79]. However, the 20(*S*) isomer showed a greater inhibitory effect. Many factors may contribute to the

Table 4 Pharmacological actions of various ginsenosides

	Ginsenosides
Antiplatelet aggregation	Ro, Rg_1, Rg_2
Fibrinolytic action	Ro, Rb_1, Rb_3, Rc, Re, Rg_1, Rg_2
Stimulation of phagocytic action	Ro, Rb_1, Rb_2, Rc, Rg_3, Rh_2, Re, Rg_2, Rh_1
Vasodilating action	Rb_1, Rd, Rg_1
Cholesterol and neutral lipid decreasing and HDL-cholesterol increasing effects	Rb_1, Rb_2, Rc
Stimulation of ACTH corticosterone secretion	Rb_1, Rb_2, Rc, Re
Stimulation of RNA polymerase, protein synthesis	Rb_1, Rc, Rg_1
Inhibition of cancer cell invasion	Rg_3
Induction of reverse transformation	Rh_2
Inhibition of tumor angiogenesis	Rb_2

multiple pharmacological effects of ginsenosides. The structural isomerism and stereoisomerism exhibited by ginsenosides increase their pharmacological diversity.

3.2 Ginsenoside Biosynthesis and Manipulation of Ginsenoside Heterogeneity

3.2.1 Ginsenoside Biosynthesis

Ginsenosides are synthesized via the isoprenoid pathway by cyclization of 2,3-oxidosqualene to give primarily oleanane dammarane triterpenoid skeletons (dammarenediol or β-amyrin). The first committed step in the synthesis of triterpenoid saponins involves the cyclization of 2,3-oxidosqualene to give one of a number of different potential products. Ginsenosides are derived from dammarane skeletons or oleanane. Dammarenyl cation produced by this cyclization forms a branching point in the ginsenoside biosynthetic pathway (Fig. 7).

The oleanane or dammarane skeleton undergoes various modifications (oxidation, substitution, and glycosylation), mediated by cytochrome P450 dependent monooxygenases, glycosyltransferases, and other enzymes, to form various protopanaxadiol-type, protopanaxatriol-type, and oleanane-type ginsenosides. Like other saponins, it is believed that the oligosaccharide chains were likely to be synthesized by the sequential addition of single sugar molecules to the aglycone [82, 83]. Compared with that of protopanaxadiol-type ginsenosides, the aglycone of protopanaxatriol-type ginsenosides (protopanaxatriol) has one more hydroxyl group at C-6, which possibly stems

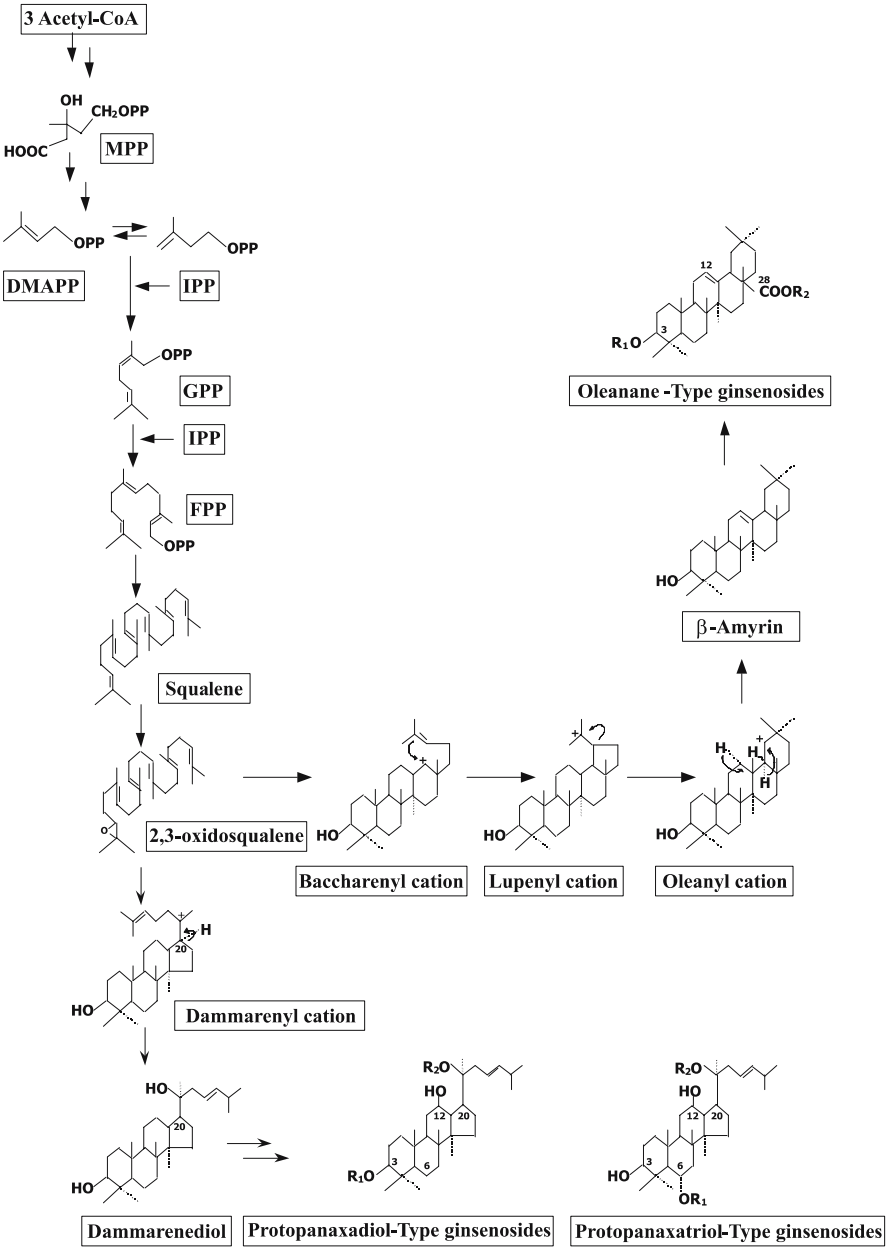


Fig. 7 The proposed ginsenoside biosynthetic pathway (modified from Refs. [80, 81])

from protopanaxadiol by oxidation. Glycosylation sites of protopanaxatriol are usually C-6 and C-20, but not C-3, at which glycosylation occurs for protopanaxadiol.

3.2.2
Manipulation of Ginsenoside Heterogeneity

Manipulation of ginsenoside heterogeneity has been performed in cell cultures, especially in *P. notoginseng* cell cultures. *P. notoginseng*, a famous traditional Chinese medicinal herb, is an important source of ginsenosides, and it has been used as a source of a healing drug and health tonic in oriental countries since ancient times. Ginsenosides, mostly protopanaxadiol-type and protopanaxatriol-type, are known as its major bioactive secondary metabolites. The main strategies for manipulation of individual ginsenoside biosynthesis are to intentionally change environmental factors in cell cultures.

3.2.2.1
Addition of Jasmonates

At present, the metabolic pathway engineering of ginseng cells for manipulation of the ginsenoside heterogeneity is very difficult, since it is not clear how each individual ginsenoside is synthesized. In a primary study, it was suggested that both the amount and the type of the ginsenoside produced by the cultured cells of *P. notoginseng* could be varied under different culture modes [124]. Elicitation of jasmonates proved to be an effective way to manipulate ginsenoside heterogeneity [84].

Different jasmonates play different roles in ginsenoside biosynthesis. Dihydromethyl jasmonate (HMJA) showed less effect than MJA on ginsenoside synthesis, and only the 100 μM concentration of HMJA increased the ginsenoside content. In contrast, MJA showed a significant effect, and more importantly, MJA changed the ratio of ginsenoside content. The content of ginsenoside Rb_1 increased much more than that of ginsenosides Rg_1 and Re did. In addition, Rd was easily detected upon the addition of MJA. The ratio of the Rb (protopanaxadiol-type) to the Rg (protopanaxatriol-type) groups of the ginsenosides increased from 0.67 (control) to 1.84 (at 100 μM MJA). In contrast, under HMJA elicitation, the ratio of Rb to Rg did not change significantly, and no Rd was detected. The results suggest that MJA is a promising compound for the manipulation of the heterogeneity of ginsenosides in *P. notoginseng* cell cultures [84].

The MJA concentration was also significant for the ginsenoside synthesis [84]. Table 5 presents the contents of different ginsenosides at MJA concentrations of 20–500 μM. MJA remarkably enhanced the ginsenoside content and altered its distribution in the cell cultures. The total ginsenoside content increased with increasing MJA concentration from 20 to 200 μM, then a slight decrease was observed at even higher concentrations of MJA. Upon addition of MJA, the ginsenoside content of the Rb group increased much more than that of the Rg group. In particular, the content of Rb_1 increased far more than

Table 5 Effects of methyl jasmonate (*MJA*) concentration on the production and distribution of individual ginsenosides

MJA concentration (μM)	Ginsenoside production (mg/L)					Rb:Rg[b]
	Rg_1	Re	Rb_1	Rd	Total[a]	
Day 12						
0	39.2 ± 1.4	34.0 ± 2.6	28.3 ± 1.9	0 ± 0	101 ± 6	0.39
0[c]	29.5 ± 1.1	29.9 ± 3.0	26.7 ± 2.4	0 ± 0	86.1 ± 6.5	0.45
20	68.0 ± 3.7	54.6 ± 1.4	114 ± 8	13.4 ± 3.3	250 ± 16	1.04
100	68.9 ± 3.6	54.6 ± 1.4	190 ± 18	23.5 ± 0.5	337 ± 24	1.72
200	68.7 ± 1.5	53.3 ± 2.2	226 ± 15	22.2 ± 5.9	370 ± 25	2.03
500	39.1 ± 0.4	26.8 ± 0.4	136 ± 10	5.99 ± 0.64	207 ± 11	2.07
Day 15						
0	25.1 ± 1.7	34.3 ± 2.3	29.1 ± 1.6	0 ± 0	88.5 ± 5.6	0.49
0[c]	27.9 ± 2.0	33.7 ± 0.8	38.3 ± 2.6	0 ± 0	99.9 ± 5.4	0.62
20	65.4 ± 11.0	61.4 ± 8.8	132 ± 16	9.12 ± 0.45	268 ± 36	1.12
100	65.9 ± 0.4	60.5 ± 0.5	195 ± 3	12.7 ± 1.2	333 ± 5	1.64
200	66.8 ± 0.0	64.7 ± 0.6	256 ± 6	15.9 ± 0.6	403 ± 7	2.06
500	36.7 ± 2.0	35.9 ± 2.1	164 ± 5	7.28 ± 0.39	244 ± 10	2.49

[a]Total content=(Rg1+Re+Rb1+Rd)
[b]Rb:Rg=(Rb1+Rd)/(Rg1+Re)
[c]The control with addition of 1mL/L ethanol, which was used for dissolving MJA

that of Rg_1 and Re, and Rd was also detected in all cases of MJA supplementation. An increase in MJA concentration from 0 to 500 μM resulted in an increase in the ratio of Rb to Rg from 0.39 to 2.07 on day 12 and from 0.49 to 2.49 on day 15. It was also observed that the ratio of Rb to Rg increased sharply with addition of 200 μM MJA, while there was no significant change for the control during the entire cultivation period (Fig. 8). The improvement of ginsenoside production and the alteration of ginsenoside distribution (heterogeneity) by jasmonate elicitation were also observed in adventitious root cultures of *P. ginseng* [85]. All those facts suggest that jasmonate as a signal transducer may activate major enzymes in the isoprenoid pathway up to dammarenediol and may also enhance key enzyme activities in the biosynthetic steps from dammarenediol to individual ginsenosides (especially Rb_1 and Rd).

The combination of MJA re-elicitation with sucrose feeding was demonstrated to be a simple and effective strategy for hyperproduction of ginsenosides and efficient manipulation of their heterogeneity in a bioreactor. The maximum cell dry weight (DW), the ginsenoside content when the cells reached their maximum DW, and the maximum ginsenoside production for the control, for MJA elicited twice and, for the combination strategy are summarized in Table 6. The maximum DW for the combination strategy was 25.1 ± 0.3 and 27.3 ± 1.5 g/L on day 17 in a flask and an airlift bioreactor (ALR), respectively, which was about 20 and 30% higher than for the control and for MJA elicited twice in both cases. Similar to MJA re-elicitation, in both cultivation vessels, the ginsenoside content was also highly enhanced with the combination strategy, and therefore higher ginsenoside production was obtained. For example, in the ALR with the combination strategy, the production of ginsenosides Rg_1, Re, Rb_1, and Rd was 118.4 ± 4.7, 117.2 ± 4.6, 290.2 ± 5.1, and 32.7 ± 8.1 mg/L, respectively, which was apparently higher

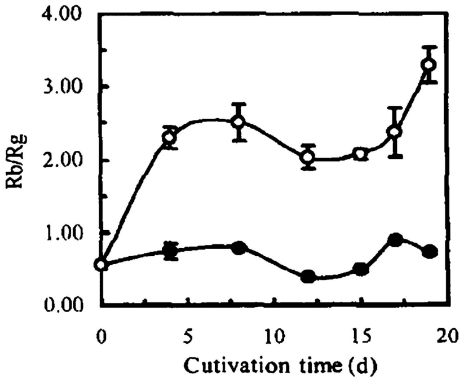


Fig. 8 Dynamic profiles of the ginsenoside Rb-to-Rg ratio in *Panax notoginseng* cell cultures. Control (*closed symbols*), methyl jasmonate (*MJA*) addition (*open symbols*)

Table 6 Effects of combination strategy on maximum dry weight (*DW*), individual ginsenoside content, and maximum production of individual ginsenosides

Cultivation conditions	Maximum DW (g/L)	Ginsenoside content (mg/100 mg DW)					Ginsenoside production (mg/L)			
		Rg_1	Re	Rb_1	Rd	Total[1]	Rg_1	Re	Rb_1	Rd
Flasks										
Control (day 15)	20.8 ± 0.8^{a}	0.24 ± 0.01^{a}	0.25 ± 0.02^{a}	0.24 ± 0.02^{a}	0^{a}	0.74 ± 0.03^{a}	50.3 ± 3.7^{a}	52.4 ± 1.0^{a}	50.9 ± 3.3^{a}	0^{a}
MJA elicited twice[2] (day 17)	18.9 ± 0.5^{b}	0.42 ± 0.01^{b}	$0.45 \pm 0.01^{b,c}$	1.17 ± 0.04^{b}	$0.11 \pm 0.03^{b,c}$	$2.15 \pm 0.07^{b,c}$	79.3 ± 4.8^{b}	85.0 ± 5.0^{b}	220.4 ± 2.2^{b}	20.8 ± 5.9^{b}
Combination strategy[3] (d 17)	25.1 ± 0.3^{c}	0.45 ± 0.01^{c}	0.46 ± 0.02^{b}	1.22 ± 0.03^{b}	0.14 ± 0.04^{b}	2.27 ± 0.05^{b}	112.9 ± 2.1^{c}	120.4 ± 2.9^{c}	306.1 ± 4.5^{c}	35.1 ± 6.9^{c}
ALR										
Control (day 15)	23.1 ± 1.6^{d}	0.21 ± 0.02^{d}	0.22 ± 0.01^{a}	0.22 ± 0.03^{a}	0^{a}	0.64 ± 0.05^{a}	48.5 ± 3.1^{a}	49.9 ± 3.4^{a}	49.8 ± 2.4^{a}	0^{a}
MJA elicited twice[2] (day 17)	21.3 ± 0.9^{a}	0.39 ± 0.02^{e}	0.42 ± 0.02^{c}	0.98 ± 0.04^{c}	0.09 ± 0.01^{c}	1.87 ± 0.10^{d}	82.1 ± 8.1^{b}	88.5 ± 8.3^{b}	209.0 ± 8.0^{b}	19.2 ± 3.8^{b}
Combination strategy[3] (day 17)	27.3 ± 1.5^{e}	$0.41 \pm 0.02^{b,e}$	$0.43 \pm 0.01^{b,c}$	1.06 ± 0.07^{d}	$0.12 \pm 0.04^{b,c}$	$2.02 \pm 0.06^{c,d}$	111.8 ± 4.7^{c}	117.2 ± 4.6^{c}	290.2 ± 5.1^{c}	32.7 ± 8.1^{c}

a, *b*, *c*, *d*, and *e* means with the same *letter* all noted in a single column are not significantly different according to Tukey's honestly significant difference multiple-comparison test with a family error rate of 0.05.

[1]Total content = (Rg_1+Re+Rb_1+Rd)

[2]MJA re-elicitation: 200 μM of MJA added on days 8 and 13, respectively

[3]Combination strategy: 200 μM of MJA added on days 8 and 13 with feeding of 10 g sucrose/L on day 13

than for the control and for MJA re-elicitation. The results show that MJA re-elicitation combined with sucrose feeding was also suitable for the bioreactor cultivation of *P. notoginseng* cells for hyperproduction of heterogeneous ginsenosides [86].

Furthermore, our laboratory has used novel chemically synthesized 2-hydroxyethyl jasmonate (HEJA) to induce the ginsenoside biosynthesis and to manipulate the product heterogeneity in cell suspension cultures of *P. notoginseng* [87]. It was interestingly found that HEJA could stimulate ginsenoside biosynthesis and change the heterogeneity more efficiently than MJA, and the activity of the Rb_1 biosynthetic enzyme, i.e., UDPG:ginsenoside Rd glucosyltransferase (UGRdGT), was also higher in the former case (Fig. 9). By investigating two signal events in the plant defense response, i.e., oxidative burst and jasmonic acid (JA) biosynthesis, the results suggest that an oxidative burst might not be involved in the jasmonate-elicited signal transduction pathway, and MJA and HEJA may induce the ginsenoside biosynthesis via induction of endogenous JA biosynthesis and key enzymes in the ginsenoside biosynthetic pathway such as UGRdGT. The information is considered useful for hyperproduction of plant-specific heterogeneous products.

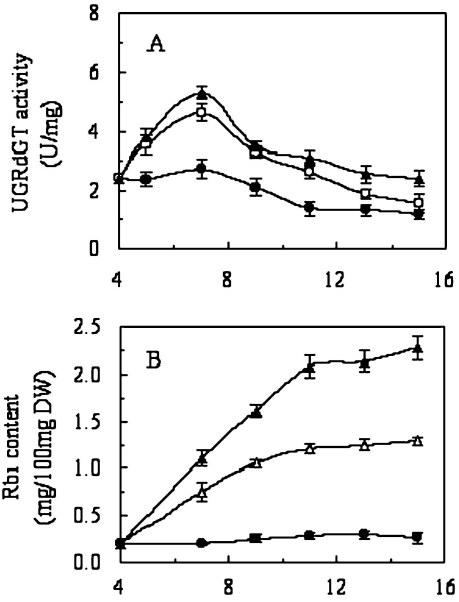


Fig. 9 **a** Dynamic changes of UDPG:ginsenoside Rd glucosyltransferase (*UGRdGT*) activity **b** and the content of ginsenoside Rb_1 for *P. notoginseng* cells with 200 μM MJA or 2-hydroxyethyl jasmonate (*HEJA*) elicited on day 4. Control (*circles*), 200 μM MJA added on day 4 (*open triangles*), 200 μM HEJA added on day 4 (*closed triangles*)

3.2.2.2 Change of Oxygen Partial Pressure

Although the oxygen requirement of plant cells is relatively modest compared with that of microbial cells, high cell density and fluid viscosity could significantly reduce the oxygen transfer efficiency in bioreactors. An alternative approach to avoid oxygen limitation in bioreactors is via manipulation of oxygen partial pressure (pO_2). Different pO_2 levels could be obtained by mixing air with different ratios of pure oxygen or nitrogen while the total aeration rate was maintained constant. Different pO_2 levels affected the distribution of ginsenosides (heterogeneity) in high-density cell cultures in 1-L ALRs [88]. On day 10, the ratio of Rb to Rg at pO_2 of 36.5 kPa is 1.8- and 1.5-fold that at pO_2 of 10.6 and 21.3 kPa, respectively, while supplementation of CO_2 at pO_2 of 10.6 and 36.5 kPa had no obvious effects on ginsenoside formation. The results imply that pO_2 may play an interesting role in ginsenoside biosynthesis via signal transduction like an oxidative burst [88].

3.2.2.3 Change of External Calcium Concentration

Calcium is considered as the most versatile intracellular messenger, and is able to couple a wide range of extracellular signals to specific responses [89]. In recent years, evidence has suggested that extracellular Ca^{2+} affects plant secondary metabolite production [90, 91]. It was observed that external calcium not only affected biosynthesis of ginsenoside Rb_1 [92], but also changed the Rb to Rg ratio (Table 7). External calcium affected the content of intracellular calcium and calmodulin (CaM) and the activities of calcium-dependent protein kinases (CDPKs) and key enzymes leading to ginsenoside heterogeneity, e.g., ginsenoside glycosyltransferases such as UGRdGT [92]. It is proposed that the effects of external calcium on the ginsenoside biosynthesis by *P. notoginseng* cells are possibly mediated via a signal transduction pathway (Fig. 10). Regulation of the external calcium concentration is considered as a useful and powerful tool for manipulating ginsenoside synthesis and its heterogeneity in a large-scale cultivation process.

3.2.2.4 Biotransformation

The distribution of various ginsenosides in ginseng cells is very different, and unfortunately the rare ginsenosides usually present higher physiological activity than the abundant ones. For example, ginsenoside Rh_2, whose content in wild ginseng is around 0.00003 (by dry weight), shows stronger potency to inhibit tumor growth than that of ginsenoside Rb_1, whose content is around 0.01. To date, it is very difficult to manipulate the accumulation of rare gin-

Table 7 Effects of external calcium concentration on the distribution of individual ginsenosides

Initial Ca^{2+} concentration (mM)	Verapamil addition or Ca^{2+} feeding	Rb:Rg[a] 0 h	24 h	48 h	72 h
0	–	0.43	0.42	0.43	0.43
3	–	0.43	0.45	0.49	0.51
8	–	0.43	0.48	0.57	0.61
13	–	0.43	0.44	0.45	0.48
3	Addition of 0.5 mM Verapamil at initial time	0.43	0.42	0.43	0.47
3	Feeding of 5 mM Ca^{2+} at 24 h	0.43	0.42	0.57	0.66
3	Feeding of 5 mM Ca^{2+} at 24 and 48 h	0.43	0.42	0.57	0.57

[a] Rb:Rg=Rb1/(Rg1+Re)

senosides in ginseng cells as their biosynthetic process is unclear. Biotransformation is a practical approach to transform highly abundant ginsenosides into rare ones by using isolated enzymes or microorganisms. Table 8 shows

Table 8 Biotransformation of ginsenosides by enzymes or microorganisms

Transformation of ginsenosides	Enzymes or microorganisms
$Rg_3 \rightarrow Rh_2$	Ginsenoside-β-glucosidase (from *Panax ginseng*) [93] *Rhizopus stolonifer* AS 3.822 [94] *Bacteroides* sp., *Fusobacterium* sp., *Bifidobacterium* sp. [95]
$Rc \rightarrow Rd$	Ginsenoside-α-arabinofuranase (from *P. ginseng*) [96]
$Rg_2 \rightarrow Rh_1$	Ginsenoside-α-L-rhamnosidase (from *Absidia* sp.39) [97]
$Rb_1 \rightarrow F_2$	Ginsenoside-β-glucosidase (from *Fusobacterium* K-60) [98]
$Rg_1, Re \rightarrow Rh_1$	Lactase (from *Penicillium sp.*) [99]
$Rb_2 \rightarrow Rd$	α-L-Arabinopyranosidase (from *Bifidobacterium breve* K-110) [100]
$Rc \rightarrow Rd$	α-L-Arabinofuranosidase (from *B. breve* K-110) [100]
$Re \rightarrow Rg_1$	Hesperidinase (from *Penicillium* sp.) [101]
$Rb_1 \rightarrow Rd$	*Curvularia lunata* AS 3.4381, *R. stolonifer* AS 3.822 [94]
$Rd \rightarrow Rg_3$	*R. stolonifer* AS 3.822 [94]

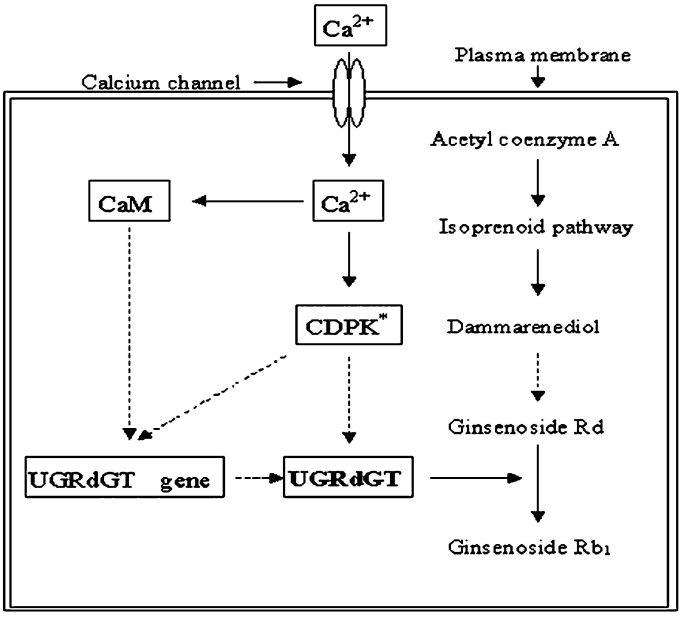


Fig. 10 A proposed signal transduction pathway regarding the effect of external Ca^{2+} on biosynthesis of ginsenoside Rb_1 by *P. notoginseng* cells. Ca^{2+} signal changes are triggered by various concentrations of external Ca^{2+}. The calcium signatures are decoded by calcium sensors, calmodulin (*CaM*) and calcium-dependent protein kinase (*CDPK*). UGRdGT, which catalyzes ginsenoside Rb_1 synthesis from Rd, is possibly modulated by the sensors in a direct or an indirect way (*dashed lines*). Changes of CDPK activity may result from increased synthesis of CDPK protein or from post-translational modification of the enzyme (*CDPK**)

some enzymes and microorganisms used in ginsenoside biotransformation. High biotransformation rates have been observed. For example, after reaction at 60 °C for 24 h, over 60% of ginsenoside Rg_3 was converted to Rh_2 by ginsenoside-β-glucosidase from ginseng [93]. After 4-day incubation on a rotary shaker (200 rpm) at 24 °C with *Curvularia lunata*, 81% of ginsenoside Rb_1 was transformed into Rd [94]. Besides hydrolyzing the ginsenosides conjugated with many sugars to that conjugated with fewer sugars, glycosylation on the ginsenosides with a few sugars is another method of ginsenoside biotransformation. The UGRdGT isolated from *P. notoginseng* cell cultures in our laboratory allowed over 80% of ginsenoside Rd to produce Rb_1 after reaction at 30 °C for 10 h with uridine 5′-diphosphoglucose. Although both isolated enzymes and microorganisms can convert ginsenosides, the products of ginsenoside biotransformation by enzymes are single ones and its incubation time is also shorter than for conversion by microorganisms. Thus, the biotransformation by enzymes is a promising approach in the manipulation of ginsenoside heterogeneity. But, its disadvantage is that another ginsenoside

(as a substrate) and the enzyme (as a biocatalyst) are necessary, which may cause a high cost especially for large-scale production.

4 Perspectives

As we gain deeper insight into the metabolic network and its interaction with the environment of biosynthetic pathways for plant secondary metabolism, more rational approaches to redirecting metabolic flux to desired secondary metabolites could be designed. By integrating molecular biology techniques with mathematical analysis tools, we can use metabolic engineering to help elucidate metabolic flux control and rational selection of targets for genetic modification [102, 103]. In the case of plant alkaloids (one of the largest groups of natural products), which provide many pharmacologically active compounds, significant progress, such as increased indole alkaloid levels, altered tropane alkaloid accumulation, elevated serotonin synthesis, reduced indole glucosinolate production, redirected shikimate metabolism, and increased cell-wall-bound tyramine formation, has been achieved by metabolic engineering applications [104–107].

Functional genomics (transcriptomics, proteomics, and metabolomics) also offer new avenues for potential manipulation of heterogeneity of plant secondary metabolites. Because not enough genomic tools are available for most plants producing interesting secondary metabolites (e.g., ginsenosides and paclitaxel), despite great progress in cDNA cloning of enzymes related to biosynthesis of paclitaxel [108], it is not surprising that virtually no such comprehensive studies have been reported. Recently, a proteomic approach was taken to analyze the proteins in opium poppy latex, which is thought to be the major site of morphine biosynthesis [109]. This type of analysis based on two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis is helpful to identify the genes required for specific cell factories that are responsible for the biosynthesis of plant secondary metabolites such as morphine. It is very important to analyze the protein itself closely related to secondary metabolism, because the DNA sequence and the expression of messenger RNA (mRNA) do not provide information of protein post-translational modification, structure, and protein–protein interaction. Almost all proteins are post-translationally modified, and then form specific structures and functions through protein–protein interaction [110]. In addition, transcriptomics tools such as differential display, expressed sequence tag databases and microarrays have also been used to investigate the biosynthesis of specific secondary metabolites, and, in particular, random sequencing of cell cDNA libraries from MJA-induced *T. cuspidata* cells

for taxoid biosynthesis has been used to isolate the entire paclitaxel pathway [108, 111–113].

Considering the network of the biosynthetic pathway of plant secondary metabolites, the same metabolite can be a member of several different pathways and may also have regulatory effects on multiple biological processes. Therefore, an individual metabolite cannot, in most cases, be unambiguously linked to a single genomic sequence [114]. Thus, the simultaneous identification and quantification of metabolites is necessary to study the dynamics of the metabolome of secondary metabolism, to analyze fluxes in secondary metabolic pathways, and to decipher the role of each metabolite following various stimuli. Linkage of functional metabolomic information to mRNA and protein expression data makes it possible to visualize the functional genomic repertoire of cells [115]. Such knowledge is believed to have great potential for manipulation of heterogeneity of plant secondary metabolites.

In the postgenomic era, the processes and strategies to manipulate plant cell cultures for heavy accumulation of desired secondary metabolites such as Tc are possibly like the following: establishment of cell cultures able to produce Tc; determination of suitable cultivation conditions, for example, elicitation with novel synthetic jasmonates [116, 117] or other stimuli which activate the genes involved in Tc biosynthesis and enhance Tc production; metabolite profiling by means of gas chromatography–mass spectrometry (MS), liquid chromatography–MS, NMR, and so on; proteomic analysis; discovery of genes related to Tc accumulation by means of cDNA–amplified fragment length polymorphism, serial analysis of gene expression and microarrays, and integration with proteome analysis data; enhancement of expression or activity of rate-limiting enzymes via transformation with selected genes alone or in combination; decrease of the flux through competitive pathways and the catabolism of Tc and prevention of feedback inhibition of a key enzyme via manipulation by transcription factors or antisense technology; and combination with engineering strategies such as pulsed electric field stimulation [118].

Until now, only a few of the these strategies have been successfully demonstrated in plant cells. Recently, the simultaneous overexpression of two genes encoding the rate-limiting upstream enzyme putrescine *N*-methyltransferase and the hyoscyamine-6β-hydroxylase of tropane alkaloid biosynthesis resulted in the highest scopolamine production ever obtained in cultivated *H. niger* hairy roots [119]. Antisense approaches and transcription factors were also successfully applied to manipulation of secondary metabolite production [120, 121]. Because transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds, the use of specific transcription factors would avoid the time-consuming step of acquiring knowledge about all enzymatic steps of a poorly characterized biosynthetic pathway [122]. For example, high-flavonol tomatoes were obtained via the heterologous expression of the maize transcription

factor genes [123]. It is expected that very efficient production of high-value-added secondary metabolites by plant cells will be possible with the advancement of functional genomic technology.

Acknowledgements W. Wang contributed to our ginsenoside heterogeneity project. Financial support from the National Natural Science Foundation of China (NSFC project nos. 30270038 and 20236040) and the Shanghai Science & Technology Commission (project no. 04QMH1410) is gratefully acknowledged. J.J.Z. also thanks the National Science Fund for Distinguished Young Scholars (NSFC project no. 20225619) and the Cheung Kong Scholars Program of the Ministry of Education of China.

References

1. Hostettmann K, Terreaux C (2000) Search for new lead compounds from higher plants. Chimia (Aarau) 54:652–657
2. Verpoorte R (1998) Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Discov Today 3:232–238
3. De Luca V, St Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5:168–173
4. Wink M (1998) Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet 75:225–233
5. Harborne JB, Baxter H (1999) The handbook of natural flavonoids, vol 1. Wiley, Chichester
6. Buckingham J (ed) (2000) Dictionary of natural products on CD. Chapman & Hall/CRC, UK
7. Ibrahim RK, Varin L (1993) Flavonoid enzymology. In: Lea PJ (ed) Methods in plant biochemistry, vol 9. Academic, London, pp 99–131
8. Facchini PJ (1999) Plant secondary metabolism: out of the evolutionary abyss. Trends Plant Sci 4:382–384
9. Osbourne AE, Wubben PJ, Melton RE, Carter JP, Daniels MJ (1998) Saponins and plant defense. In: Romeo TJ, Downum KR, Verpoorte R (eds) Phytochemical signal and plant-microbe interactions. Plenum, New York, pp 1–16
10. Chappell J (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu Rev Plant Physiol Plant Mol Biol 46:521–547
11. Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants. ASPB, Rockville, MD, pp 1250–1268
12. McGarvey DJ, Croteau R (1995) Terpenoid metabolism. Plant Cell 7:1015–1026
13. Kingston DGI (2001) Taxol, a molecule for all seasons. Chem Commun 867–880
14. Zheng GZ, Yang CFL (1994) Sanchi (Punux notoginseng): biology and application. Science, Beijing (in Chinese)
15. Sticher O (1998) Getting to the root of ginseng. CHEMTECH 28:26–32
16. Stafford AM, Pazoles CJ, Siegel S, Yeh L-A (1998) Plant cell culture: a vehicle for drug discovery. In: Harvey AL (ed) Advances in drug techniques. Wiley, New York, pp 53–64
17. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumour agents VI. The isolation and structure of taxol, a novel antileukemic and antitumour agent from Taxus brevifolia. J Am Chem Soc 93:2325–2327

18. Miller RW, Powell RG, Smith CR, Arnold E, Clardy J (1981) Antileukemic alkaloids from Taxus wallichiana Zucc. J Org Chem 46:1469–1474
19. Witherup KM, Look SA, Stasko MW, Ghiorzi TJ, Muschik GM (1990) Taxus spp.: needles contain amounts of taxol comparable to the bark of Taxus brevifolia: analysis and isolation. J Nat Prod 53:1249–1255
20. Fett-Neto AG, DiCosmo F (1992) Distribution and amount of taxol in different shoot parts of Taxus cuspidata. Planta Med 58:464–466
21. ElSohly HN, Croom ED, Kopycki WJ, Joshi AS, ElSohly MA, McChesney JD (1995) Concentrations of taxol and related taxanes in the needles of different Taxus cultivars. Phytochem Anal 6:149–156
22. Singh B, Gujral RK, Sood RP, Duddeck H (1997) Constituents from Taxus species. Planta Med 63:191–192
23. Strobel GA, Ford E, Li JY, Sears J, Sidhu RS, Hess WM (1999) Seimatoantlerium tepuiense gen. nov., a unique epiphytic fungus producing taxol from the Venezuelan-Guayana system. Appl Microbiol 22:426–433
24. Wang J, Li G, Lu H, Zheng Z, Huang Y, Su W (2000) Taxol from Tubercularia sp. strain 333 TF5, an endophytic fungus of Taxus mairei. FEMS Microbiol Lett 193:249–253
25. Shrestha K, Strobel GA, Prakash S, Gewali M (2001) Evidence for paclitaxel from three new endophytic fungi of Himalayan yew of Nepal. Planta Med 6 7:374–376
26. Baloglu E, Kingston DGI (1999) The taxane diterpenoids. J Nat Prod 62:1448–1472
27. Sledge GW (2003) Gemcitabine combined with paclitaxel or paclitaxel/trastuzumab in metastatic breast cancer. Semin Oncol 30:19–21
28. O'Brien MER, Splinter T, Smit EF, Biesma B, Krzakowski M, Tjan-Heijnen VCG, Van Bochove A, Stigt J, Smid-Geirnaerdt MJA, Debruyne C, Legrand C, Giaccone G (2003) Carboplatin and paclitaxol (Taxol) as an induction regimen for patients with biopsy-proven stage IIIA N2 non-small cell lung cancer: an EORTC phase II study (EORTC 08958). Eur J Cancer 39:1416–1422
29. Guéritte F (2001) General and recent aspects of the chemistry and structure-activity relationships of taxoids. Curr Pharm Design 7:1229–1249
30. Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly invitro by taxol. Nature 277(5698):665–667
31. Kingston DGI (2000) Recent advances in the chemistry of taxol. J Nat Prod 63:726–734
32. Shigemori H, Kobayashi J (2004) Biological activity and chemistry of taxoids from the Japanese yew, Taxus cuspidate. J Nat Prod 67:245–256
33. Eisenreich W, Menhard B, Hylands PJ, Zenk MH, Bacher A (1996) Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin. Proc Natl Acad Sci USA 93:6431–6436
34. Eisenreich W, Rohdich F, Bacher A (2001) Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci 6:78–84
35. Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 295:517–524
36. Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant 101:643–652
37. Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50:47–65

38. Koepp AE, Hezari M, Zajicek J, Stofer-Vogel B, LaFever RE, Lewis NG, Croteau R (1995) Cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene is the committed step of taxol biosynthesis in Pacific yew. J Biol Chem 270:8686–8690
39. Hezari M, Lewis NG, Croteau R (1995) Purification and characterization of taxa-4(5),11(12)-diene synthase from Pacific yew (Taxus brevifolia) that catalyses the first committed step of Taxol biosynthesis. Arch Biochem Biophys 322:437–444
40. Hezari M, Ketchum REB, Gibson DM, Croteau R (1997) Taxol production and taxadiene synthase activity in Taxus canadensis cell suspension cultures. Arch Biochem Biophys 337:185–190
41. Dong HD, Zhong JJ (2001) Significant improvement of taxane production in suspension cultures of Taxus chinensis by combining elicitation with sucrose feed. Biochem Eng J 8:145–150
42. Hefner J, Rubenstein SM, Ketchum REB, Gibson DM, Williams RM, Croteau R (1996) Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-diene-5α-ol: the first oxygenation step in taxol biosynthesis. Chem Biol 3:479–488
43. Jennewein S, Rithner CD, Williams RM, Croteau RB (2001) Taxol biosynthesis: Taxane 13α-hydroxylase is a cytochrome P450-dependent monooxygenase. Proc Natl Acad Sci USA 98:13595–13600
44. Walker KD, Ketchum REB, Hezari M, Gatfield D, Goleniowski M, Barthol A, Croteau R (1999) Partial purification and characterization of acetyl coenzyme A: taxa-4(20),11(12)-dien-5α-ol-*o*-acetyl-transferase that catalyses the first acetylation step of taxol biosynthesis. Arch Biochem Biophys 464:273–279
45. Jennewein S, Rithner CD, Williams RM, Croteau R (2003) Taxoid metabolism: taxoid 14β-hydroxylase is a cyto-chrome P450-dependent monooxygenase. Arch Biochem Biophys 413:262–270
46. Chau M, Jennewein S, Walker K, Croteau R (2004) Taxol biosynthesis: molecular cloning and characterization of a cytochrome P450 taxoid 7β-hydroxylase. Chem Biol 11:663–672
47. Floss HG, Mocek U (1995) Biosynthesis of taxol. In: Suffness M (ed.) Taxol science and applications. CRC, Boca Raton, pp 191–298
48. Kingston DGI, Molinero AA, Rimoldi JM (1993) The taxane diterpenoids. Prog Chem Org Nat Prod 61:1–206
49. Della Casa De Marcano DP, Halsall TG (1970) Crystallographic structure determination of the diterpenoid baccatin-V, a naturally occurring oxetane with a taxane skeleton. Chem Commum 1382–1383
50. Guéritte-Voegelein F, Guénard D, Potier P (1987) Taxol and derivatives: a biogenetic hypothesis. J Nat Prod 50:9–18
51. Walker K, Long R, Croteau R (2002) The final acylation step in taxol biosynthesis: cloning of the taxoid C13-side-chain N-benzoyltransferase from Taxus. Proc Natl Acad Sci USA 99:9166–9171
52. Walker K, Croteau R (2001) Taxol biosynthetic genes. Phytochemistry 58:1–7
53. Chau M, Croteau R (2004) Molecular cloning and characterization of a cytochrome P450 taxoid 2a-hydroxylase involved in Taxol biosynthesis. Arch Biochem Biophy 427:48–57
54. McCaskill D, Croteau R (1999) Isopentenyl diphosphate is the terminal product of the deoxyxylulose-5-phosphate pathway for terpenoid biosynthesis in plants. Tetrahedron lett 40:653–656

55. Choi HK, Kim SI, Son JS, Hong SS, Lee HS, Lee HJ (2000) Enhancement of paclitaxel production by temperature shift in suspension culture of Taxus chinensis. Enzyme Microb Technol 27:593–598
56. Bai J, Kitabatake M, Toyoizumi K, Fu L, Zhang S, Dai J, Sakai J, Hirose K, Yamori T, Tomida A, Tsuruo T, Ando M (2004) Production of biologically active taxoids by a callus culture of Taxus cuspidate. J Nat Prod 67:58–63
57. Ketchum REB, Rithnerb CD, Qiua D, Kima YS, Williamsb RM, Croteaua RB (2003) Taxus metabolomics: methyl jasmonate preferentially induces production of taxoids oxygenated at C-13 in Taxus x media cell cultures. Phytochemistry 62:901–909
58. Ketchum REB, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension cultures of Taxus following elicitation with methyl jasmonate. Biotech Bioeng 62:97–105
59. Veeresham C, Mamatha R, Prasad Babu Ch, Srisilam K, Kokate CK (2003) Production of taxol and its analogues from cell cultures of Taxus wallichiana. Pharm Biol 41:426–430
60. Brincat MC, Gibson DM, Shuler ML (2002) Alterations in taxol production in plant cell culture via manipulation of the phenylalanine ammonia lyase pathway. Biotechnol Prog 18:1149–1156
61. Dai JU, Cui J, Zhu WH, Guo HZ, Ye M, Hu Q, Zhang DY, Zheng JH, Guo D (2002) Biotransformation of 2α-, 5α-, 10β-, 14β-tetra-tetraacetoxy-4(20), 11-taxadiene by cell suspension cultures of Catharanthus roseus. Planta Med 68:1113–1117
62. Dai JG, Guo HZ, Ye M, Zhu WH, Zhang DY, Hu Q, Han J, Zheng JH, Guo DA (2003) Biotransformation of 4(20),11-taxadienes by cell suspension cultures of Platycodon grandiflorum. J Asian Nat Prod Res 5:5–10
63. Dai JG, Zhang SJ, Sakai J, Bai J, Oku Y, Ando M (2003) Specific oxidation of C-14 oxygenated 4(20), 11-taxadienes by microbial transformation. Tetrahedron Lett 44:1091–1094
64. Hu SH, Tian XF, Zhu WH, Fang QC (1996) Biotransformation of 2α-, 5α-, 10β-, 14β-tetra-tetraacetoxy-4(20), 11-taxadiene by the fungi Cunninghamella elegans and Cunninghamella echinulata. J Nat Prod 59:1006–1009
65. Hu SH, Tian XF, Zhu WH, Fang QC (1996) Microbial transformation of taxoids: Selective deacetylation and hydroxylation of 2α-, 5α-, 10β-, 14β-tetra-acetoxy-4(20),11-taxadiene by the fungus Cunninghamella echinulata. Tetrahedron 52:8739–8746
66. Dai JG, Ye M, Guo HZ, Zhu WH, Zhang DO, Hu Q, Zheng JH, Guo D (2002) Regio- and stereo-selective biotransformation of 2α-,5α-,10β-, 14β-tetra-acetoxy-4(20), 11-taxadiene by Ginkgo cell suspension cultures. Tetrahedron 58:5659–5668
67. Hu SH, Tian XF, Zhu WH, Fang QC (1997) Biotransformation of some taxoids with oxygen substituent at C-14 by Cunninghamella echinulata. Biocatal Biotransform 14:241–250
68. Patel RN (1998) Tour de paclitaxel: Biocatalysis for semisynthesis. Annu Rev Microbiol 52:361–395
69. Patel RN, Banerjee A, Nanduri V (2000) Enzymatic acetylation of 10-deacetylbaccatin III to baccatin III by C-10 deacetylase from Nocardioides luteus SC 13913. Enzyme Microb Technol 27:371–375
70. Hanson RL, Kant J, Patel RN (2004) Conversion of 7-deoxy-10-deacetylbaccatin-III into 6-alpha-hydroxy-7-deoxy-10-deacetylbaccatin-III by Nocardioides luteus. Biotechnol Appl Biochem 39:209–214

71. Huang Q, Roessner CA, Croteau R, Scotta AI (2001) Engineering Escherichia coli for the synthesis of taxadiene, a key intermediate in the biosynthesis of Taxol. Bioorg Med Chem 9:2237–2242
72. Besumbes Ó, Sauret-Güeto S, Phillips MA, Imperial S, Rodriguez-Concepción M, Boronat A (2004) Metabolic engineering of isoprenoid biosynthesis in Arabidopsis for the production of taxadiene, the first committed precursor of Taxol. Biotechnol Bioeng 88:168–175
73. Soldati F, Sticher O (1980) HPLC separation and quantitative determination of ginsenosides from Panax ginseng, Panax quinquefolium and from ginseng drug preparations. Planta Med 39:348–357
74. Banthorpe DV (1994) Terpenoids. In: Mann J (ed) Natural products. Longman, Essex, UK, pp 331–339
75. Shibata S (2001) Preventing activities of ginseng saponins and some related triterpenoid compounds. J Korean Med Sci 16:S28–37
76. Odashima S, Ohta T, Kohno H, Matsuda T, Kitagawa I, Abe H, Arichi S (1985) Control of phenotypic expression of cultured B16 melanoma cells by plant glycosides. Cancer Res 45:2781–2784
77. Kim YS, Kim DS, Kim SI (1998) Ginsenoside Rh_2 and Rh_3 induce differentiation of HL-60 cells into granulocytes: Modulation of protein kinase C isoforms during differentiation by ginsenoside Rh_2. Int J Biochem Cell Biol 30:327–338
78. Islam MR, Mahdi JG, Bowen ID (1997) Pharmacological importance of stereochemical resolution of enantiomeric drugs. Drug Saf 17:149–165
79. Kudo K, Tachikawa E, Kashimoto T, Takahashi E (1998) Properties of ginseng saponin inhibition of catecholamine secretion in bovine adrenal chromaffin cells. Eur J Pharmacol 341:139–44
80. Haralampidis K, Trojanowska M Osbourn AE (2002) Biosynthesis of triterpenoid saponins in plants. Adv Biochem Eng Biotechnol 75:31–49
81. Kushiro T, Ohno Y, Shibuya M, Ebizuka Y (1997) In vitro conversion of 2,3-oxidosqualene into dammarenediol by Panax ginseng microsomes. Biol Pharm Bull 20:292–294.
82. Paczkowski C, Wojciechowski ZA (1994) Glucosylation and galactosylation of diosgenin and solasodine by soluble glycosyltransferase(s) from Solanum-melongena leaves. Phytochemistry 35:1429–1434
83. Wojciechowski ZA (1975) Biosynthesis of oleanolic acid glycosides by subcellular fraction of Calendular officinalis seedlings. Phytochemistry 14:1749–1753
84. Wang W, Zhong JJ (2002) Manipulation of ginsenoside heterogeneity in cell cultures of Panax notoginseng by addition of jasmonates. J Biosci Bioeng 93:48–53
85. Yu KW, Gao W, Hahn EJ, Paek KY (2002) Jasmonic acid improves ginsenoside accumulation in adventitious root culture of Panax ginseng C.A. Meyer. Biochem Eng J 11:211–215
86. Wang W, Zhang ZY, Zhong JJ (2005) Enhancement of ginsenoside biosynthesis in high density cultivation of Panax notoginseng cells by various strategies of methyl jasmonate elicitation. Appl Microbiol Biotechnol 67:752–758
87. Wang W (2004) Efficient induction of ginsenoside biosynthesis and manipulation of ginsenoside heterogeneity in cell suspension cultures of Panax notoginseng by addition of jasmonates. PhD thesis, ECUST, Shanghai
88. Han J, Zhong JJ (2003) Effects of oxygen partial pressure on cell growth and ginsenoside and polysaccharide production in high density cell cultures. Enzyme Microb Technol 32:498–503

89. Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell 11:691–706
90. Piñol MT, Palazón J, Cusidó RM, Ribó M (1999) Influence of calcium ion-concentration in the medium on tropane alkaloid accumulation in Datura stramonium hairy roots. Plant Sci 141:41–49
91. Nakao M, Ono K, Takio S (1999) The effect of calcium on flavanol production in cell suspension cultures of Polygonum hydropiper. Plant Cell Rep 18:759–776
92. Yue CJ, Zhong JJ (2005) Impact of external calcium and calcium sensors on ginsenoside Rb_1 biosynthesis by Panax notoginseng cells. Biotechnol Bioeng 89:444–452
93. Zhang C, Yu H, Bao Y, An L, Jin F (2001) Purification and characterization of ginsenoside-β-glucosidase from ginseng. Chem Pharm Bull 49:795–798
94. Dong A, Ye M, Guo H, Zheng H, Guo J (2003) Microbial transformation of ginsenoside Rb_1 by Rhizopus stolonifer and Curvularia lunata. Biotechnol Lett 25:339–344
95. Bae EA, Han MJ, Kim EJ, Kim DH (2004) Transformation of ginseng saponins to ginsenoside Rh_2 by acids and human intestinal bacteria and biological activities of their transformants. Arch Pharm Res 27:61–67
96. Zhang C, Yu H, Bao Y, An L, Jin F (2002) Purification and characterization of ginsenoside-α-arabinofuranase hydrolyzing ginsenoside Rc into Rd from the fresh root of Panax ginseng. Process Biochem 37:793–798
97. Yu H, Gong J, Zhang C, Jin F (2002) Purification and characterization of ginsenoside-α-L-rhamnosidase. Chem Pharm Bull 50:175–178
98. Park SY, Bae EA, Sung JH, Lee SK, Kim DH (2001) Purification and characterization of ginsenoside Rb_1-metabolizing β-glucosidase from Fusobacterium K-60, a human intestinal anaerobic bacterium. Biosci Biotechnol Biochem 65:1163–1169
99. Ko SR, Suzuki Y, Choi KJ, Kim YH (2000) Enzymatic preparation of genuine prosapogenini, 20(S)-ginsenoside Rh_1, from ginsenosides Re and Rg_1. Biosci Biotechnol Biochem 64:2739–2743
100. Shin HY, Park SY, Sung JH, Kim DH (2003) Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from Bifidobacterium breve K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb_2 and Rc. Appl Environ Microbiol 69:7116–7123
101. Ko SR, Choi KJ, Uchida K, Suzuki Y (2003) Enzymatic preparation of ginsenosides Rg_2, Rh_1, and F_1 from protopanaxatriol-type ginseng saponin mixture. Planta Med 69:285–286
102. Stephanopoulos GN, Aristidou AA, Nielsen JE (1998) Metabolic engineering: principles and methodologies. Academic, New York
103. Nielsen J (ed) (2001) Metabolic engineering. Advances in Biochemical Engineering and Biotechnology, vo1 73. Springer, Berlin Heidelberg New York
104. Yun DJ, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic Atropa belladonna with an improved alkaloid composition. Proc Natl Acad Sci USA 89:11799–11803
105. Sato F, Hashimoto T, Hachiya A, Tamura K, Choi KB, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci USA 98:367–372
106. Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annu Rev Plant Physiol Plant Mol Biol 52:29–66
107. Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004) Metabolic engineering of the indole pathway in Catharanthus roseus hairy roots and increased accumulation of tryptamine and serpentine. Metabol Eng 6:268–276

108. Jennewein S, Wildung MR, Chau M, Walker K, Croteau R (2004) Random sequencing of an induced Taxus cell cDNA library for identification of clones involved in Taxol biosynthesis. Proc Natl Acad Sci USA 101:9149–9154
109. Decker G, Wanner G, Zenk MH, Lottspeich F (2000) Characterization of proteins in latex of the opium poppy (Papaver somniferum) using two-dimensional gel electrophoresis and microsequencing. Electrophoresis 21:3500–3516
110. Hirano H, Islam, Kawasaki H (2004) Technical aspects of functional proteomics in plants. Phytochemistry 65:1487–1498
111. Yamazaki M, Saito K (2002) Differential display analysis of gene expression in plants. Cell Mol Life Sci 59:1246–1255
112. Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA (2002) A genomics approach to the early stages of triterpene saponin biosynthesis in Medicago truncatula. Plant J 32:1033–048
113. Guterman I, Shalit M, Menda N, Piestun D, Dafny-Yelin M, Shalev G, Bar E, Davydov O, Ovadis M, Emanuel M, Wang J, Adam Z, Pichersky E, Lewinsohn E, Zamir D, Vainstein A, Weiss D (2002) Rose scent: genomics approach to discovering novel floral fragrance-related genes. Plant Cell 14:2325–2338
114. Schwab W (2003) Metabolome diversity: too few genes, too many metabolites? Phytochemistry 62:837–849
115. Bino RJ, Hall RD, Fiehn O, Kopka J, Saito K, Draper J, Nikolau BJ, Mendes P, Roessner-Tunali U, Beale MH, Trethewey RN, Lange BM, Wurtele ES, Sumner LW (2004) Potential of metabolomics as a functional genomics tool. Trends Plant Sci 9:418–425
116. Qian ZG, Zhao ZJ, Tian WH, Xu Yf, Zhong JJ, Qian XH (2004) Novel synthetic jasmonates as highly efficient elicitors for taxoid production by suspension cultures of Taxus chinensis. Biotechnol Bioeng 86:595–599
117. Qian ZG, Zhao ZJ, Xu YF, Qian XH, Zhong JJ (2004) Novel chemically synthesized hydroxyl-containing jasmonates as powerful inducing signals for plant secondary metabolism. Biotechnol Bioeng 86:809–816
118. Ye H, Huang LL, Chen SD, Zhong JJ (2004) Pulsed electric field stimulates plant secondary metabolism in suspension cultures of Taxus chinensis. Biotechnol Bioeng 88:788–795
119. Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in Hyoscyamus niger hairy root cultures. Proc Natl Acad Sci USA. 101:6786–6791
120. Chintapakorn Y, Hamill JD (2003) Antisense-mediated downregulation of putrescine N-methyltransferase activity in transgenic Nicotiana tabacum L. can lead to elevated levels of anatabine at the expense of nicotine. Plant Mol Biol 53:87–105
121. Van der Fits L, Memelink J (2000) ORCA3, a jasmonate responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297
122. Gantet P, Memelink J (2002) Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. Trends Pharmacol Sci 23:563–569
123. Bovy A, de Vos R, Kemper M, Schijlen E, Pertejo MA, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell 14:2509–2526
124. Zhong JJ (1999) High-density cell cultivation and manipulation of heterogeneity of plant secondary metabolites. In: Proceedings of the APBioChEC, Phuket, Thailand, 1999