Обзорные статьи

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RECENT ADVANCES IN PLANT BIOTECHNOLOGY AND GENETIC ENGINEERING FOR PRODUCTION OF SECONDARY METABOLITES

For a long time people are using plants not only as crop cultures but also for obtaining of various chemicals. Currently plants remain one of the most important and essential sources of biologically active compounds in spite of progress in chemical or microbial synthesis. In our review we compare potentials and perspectives of modern genetic engineering approaches for pharmaceutical biotechnology and give examples of actual biotechnological systems used for production of several promising natural compounds: artemisinin, paclitaxel and scopolamine.

Introduction. One of the most important tasks for modern genetic engineering, biotechnology and pharmacology is search or creation of systems for high-scale obtaining of valuable natural products – complex organic compounds produced by living organisms. Since the earliest time people used plants not merely as food crops but additionally as sources of various chemicals: pharmaceuticals, insecticides, food supplements, dyes etc. Currently plants remain an essential provider of biologically-active compounds in spite of development of chemical or microbial technologies. A number of inherent advantages make plants the central and highly perspective object in natural product biosynthesis researches e.g. a) ecological and pharmacological safety; b) high native biosynthetic capabilities including multistep stereospecific synthesis of complex organic molecules, eukaryotic type of biopolymer synthesis and processing; c) possibilities of scaling up of valuable compound production using natural potential of plant systems; and d) economical values.

For primary classification of pharmacologically valuable plant natural substances one can define a group of mainly low molecular weight compounds, including first of all plant secondary metabolites, and a group of proteins and peptides with high molecular weight which are the products of heterological expression of foreign genes in plant cells.

All biochemical processes in plant cell can be conditionally classified as primary and secondary metabolism. Compounds and processes which are necessary for growth, development and breeding belong to primary metabolism. It includes mainly the metabolism of proteins, nucleic acids, carbohydrates and lipids. On the other hand, biosynthesis and catabolism of variable pigments, alkaloids, terpenes, phenolics belong to secondary metabolism. All these substances considered to be not directly essential for plant cell life, and their function in plants is not always clear [1, 2]. The majority of secondary compounds are considered to be participating in plant-environment interactions: they defense plants from pathogens, pests or herbivores, serve as attractants, have allelopathic, photo-protective or light-harvesting functions [1–4]. It is not surprising that most of them have a strong influence upon animal and human organism. Numerous examples of pharmaceutical applications of plant secondary metabolites are given in recent reviews [5–9].

People studied pharmacological properties of secondary compounds since great antiquity: mentions of medicinal applications of alkaloid-containing plant were found among Chinese, Mesopotamia and, later, India ancient sources dated 3000–1000 B. C. [10]. Organic synthesis progress at the close of XIX century and development of chromatographic separation protocols in the first half of XX century allowed isolation and identification of numerous organic substances responsible for pharmacological activities of plant extracts. However, in spite of considerable success in modern organic synthesis, plant's ability to form biologically active stereoisomers often makes them the unique and essential source of pharmacologically-valuable natural products. Moreover, considerable part of synthetic pharmaceuticals has been developed as modifications of natural substances of plant origin. As the experts estimate, in USA nearly 50 % of drugs for cancer chemotherapy are derivatives of plant extract components [11]. One should remark that searching of the optimal balance between drug efficiency and toxicity in the recent years brought scientists again to substances isolated from natural sources, first of all from plants [12].

In recent years an intensive work has been carried out on screening of biological activity and structural diversity of secondary metabolites. Nevertheless the biosynthetic potential of plant cells is considered to be not even half exhausted – a total amount of substances produced by plants was estimated in range about 500 thousands [13, 14]. Actual models suggest correlation between evolution of secondary metabolism in plants and reciprocal adaptation of pests or pathogens leading to divergence and stimulating biodiversity in the both groups [1].

The majority of secondary biosynthetic pathways are multistep enzymatic processes with complex and delicate regulation mechanisms on transcriptional and/or posttranscriptional level. Segregation of intermediates inside of single plant cell or their transport between different parts of the whole plant often occurs. All these factors make investigation and, especially, controlled biotechnological production of secondary compounds an extremely complicated task.

Classification of secondary metabolites may be based on the chemical structure or biological characteristics of substances. In general, three big groups of secondary compounds can be assigned: terpenes, phenolics and alkaloids, which include the main part of currently identified compounds. Their number is estimated to be from more than 50 000 structures to about 100 000 [2, 14–16].

Many terpenes exhibit strong pharmacological activities against a number of human diseases. Among them we can mention cardenolides of *Digitalis* sp. [17], glycyrrhizin extracted from the licorice root and calanolides from *Calophyllum* with anti-HIV activities [18], antibacterial shikonin from *Lithospermum erythrorhizon* [19], monoterpenoid alkaloid camptothecin isolated from *Camptotheca acuminata* and *Nothapodytes foetida* [20, 21], artemisinin from *Artemisia annua* used for malaria treatment and having additionally cytotoxic features [22, 23], and many others. In recent publications Morimoto et al. reported about successful studies of cannabinoid biosynthesis: 5 enzymes were characterized and the corresponding genes were cloned [24, 25]. Heterologous expression of tetrahydrocannabinolic acid synthase gene resulted in formation of tetrahydrocannabinolic acid (precursor of tetrahydrocannabinol) from cannabigerolic acid [24]. This gene was later expressed in *Pichia pastoris* cells. High level of enzymatic activity (app. 1.3 nkat/L) was detected in culture medium [26].

In spite of impressive scope and wide range of researches, only several secondary biosynthetic pathways have been studied in details on the enzymatic and gene levels. In our manuscript we will focus on these examples. Evidently, the frame of this publication does not allow performing a thorough review of all plant secondary metabolism research areas. Therefore we will discuss here biotechnological systems developed for production of certain valuable and perspective natural products.

Artemisinin production. Artemisinin from *A. annua* is currently one of the most effective antimalarial drugs recommended by WHO during short-course artemisinin-based combination therapy [27]. Low content of artemisinin in plants (0.01–1 % DW) and ever-growing demand for artemisinin-containing pharmaceuticals stimulated studies on biosynthetic pathway of this compound formation and attempts to enhance its accumulation in plant systems [28]. Total organic

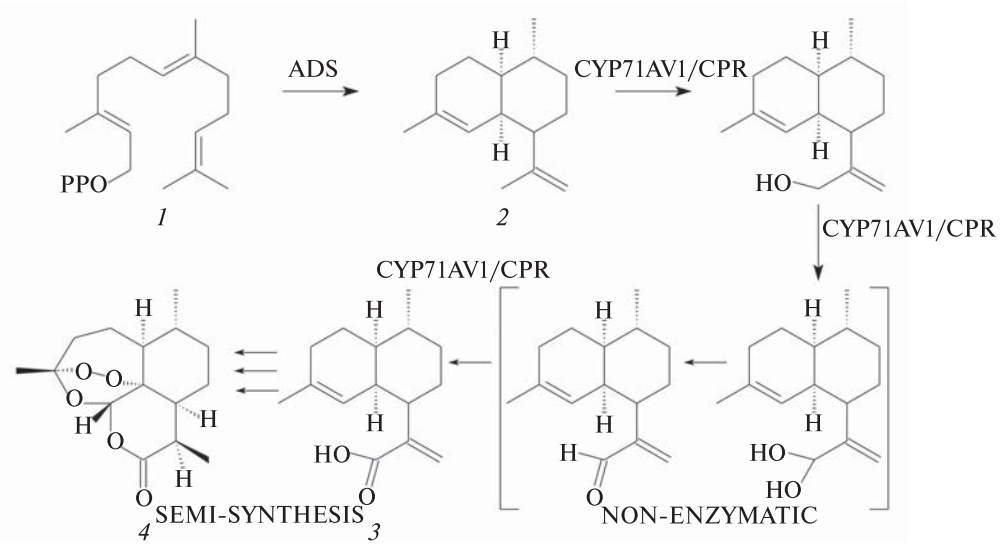

Fig. 1. Part of artemisinin biosynthesis pathway in *S. cerevisiae* (strain expressing amorphadiene synthase gene (ADS), cytochrome P450 monooxygenase (CYP71AV1) and NADPH: cytochrome P450 oxidoreductase (CPR) [35]: *1* – farnesyl pyrophosphate; *2* – amorpha-4,11-diene; *3* – artemisinic acid; *4* – artemisinin

synthesis of artemisinin was found to be very difficult and costly process [29]. More perspective were approaches on improvement of artemisinin production in plant tissue under salinity stress conditions [30].

Numerous studies have been carried out in order to obtain artemisinin from plant cell culture systems by selection of a highly productive line, supplying with precursors or elicitation [28, 31]. Additionally a hairy root culture of *A. annua* was established [32].

Cloning of several terpene biosynthesis genes like cotton farnesyl diphosphate synthase and its overexpression in *A. annua* hairy roots resulted in three- to four-fold higher yield of artemisinin [33]. Redirection of amorpha-4,11-diene synthase and farnesyl diphosphate synthase to the plastids in transgenic *Nicotiana tabacum* allowed to enhance considerably accumulation of one of the artemisinin precursors, amorpha-4,11-diene [34].

The most promising way to scale up the production of artemisinin was cloning and heterologous expression of genes coding for several consequent enzymes of mevalonate pathway (amorpha-4,11-diene synthase, cytochrome P450 monooxygenase (CYP71AV1), cytochrome P450 oxidoreductase) from *A. annua* in *Saccharomyces cerevisiae* strain (Fig. 1). As a result, 100 mg/L of artemisinic acid, direct precursor of artemisinin, were synthesized in the course of three-step reaction from native yeast intermediate metabolite farnesyl pyrophosphate. Its further conversion to artemisinin is not complex [35, 36]. Production of artemisinic acid from *S. cerevisiae* in bioreactor increased recently 25-fold and reached up to 2.5 g/L [37]. This example demonstrates efficiency of the present strategy of secondary pathway genetic engineering comprising characterization and cloning of respective genes, regulator elements and correct choice of heterologous expression system.

Paclitaxel production. Perhaps one of the most famous cytotoxic natural compounds discovered during the last decades was diterpene amid paclitaxel also known as taxol. Its antitumour activity as a component of *Taxus brevifolia* extract is known since 1965; in 1972 the chemical structure of taxol was elucidated [38]. In 1992 Taxol® was registered and appeared in the world pharmaceutical market. Numerous clinical trials proved its efficiency against several types of cancer currently making taxol one of the most perspective anticancer drugs. Cytotoxic effect of paclitaxel is based on cell division blocking by microtubules stabilization [39, 40].

Ever-growing demand for paclitaxel and its low content in wood of slowly growing yew-trees (about 0.03 % d. w. in *T. brevifolia*– bark of sever-

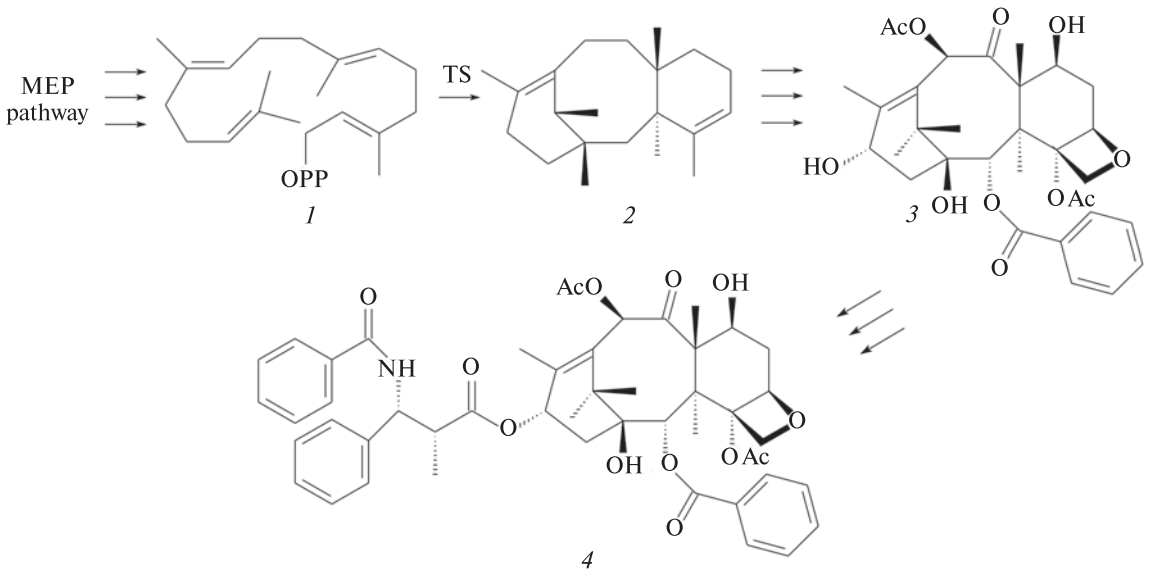

Fig. 2. Selected stages of paclitaxel biosynthesis: *1* – geranylgeranyl pyrophosphate; *2* – taxa-4(5),11(12)-diene; *3* – baccatin III; *4* – paclitaxel; TS – taxadiene synthase. Numerous arrows indicate more than one step

al hundred thousands of yew-trees needs to be extracted to supply world year demand for paclitaxel) stimulated researches on chemical and biotechnology synthesis of this compound. More than 300 relative compounds have been isolated and characterised from different *Taxus* species up to now [41].

The total chemical synthesis of paclitaxel was found to be very complex process too expensive for commercial production. Partial biosynthesis of paclitaxel and its more active derivatives like Taxotere® from precursors (for example, baccatin III) appeared more perspective. Baccatin III was isolated from yew needles that did not destroy trees and extended the source of raw materials [40, 42].

High value of paclitaxel and its extremely low natural supply became a prerequisite for numerous projects on selection of highly productive *Taxus* cell lines and enhancing of paclitaxel biosynthesis in cell cultures. Results of these studies were summarized in recent publications [43, 44]. Manipulation with cultural medium composition in combination with efficient selection allowed in a number of cases accumulation of paclitaxel in cells up to 0.03–0.05 % d. w. that is comparable or even surpasses the metabolite level in *T. brevifolia* bark [45, 46]. Further investigation proved efficiency of elicitation for taxoid biosynthesis stimulation because a number of important enzymes of terpene pathway (for instance geranylgeranyl diphosphate synthase and taxadiene synthase) are jasmonate inducible [47, 48].

Tabata reported that development of *Taxus* cell suspension selection, cultivation and elicitation protocol resulted in stable paclitaxel production up to 295 mg/L [49]. Multiple jasmonate treatments in bioreactor increased taxoid yield in cell suspensions up to 612 mg/L [50]. Companies of Phyton Catalytic Inc. (USA) and Samyang Genex (South Korea) informed about commercial isolation of paclitaxel from cell cultures [16, 39].

Two alternative pathways of terpene biosynthesis have been described at present time. Both pathways lead to production of common terpene precursors (dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP)) which can be transformed in more complex molecules in the course of further conversions. The classic mevalonate pathway (MVA) which functions in the cytosol initially was assumed to be the sole source of the terpenoid precursors IPP and DMAPP. It supplies the precursors for production of sesquiterpenes and triterpenes. Alternative pathway named after the first committed precursor, 2-*C*-methyl-D-erythritol-4-phosphate (MEP) is localized in plastids and is generally used to supply precursors

for the production of monoterpenoids, diterpenoids and tetraterpenoids [1, 51]. Moreover, recent studies showed possibilities for interchanges between intermediates of the both pathways [52]. Experiments on inhibition of IPP transport from cytoplasm to plastids demonstrated that some IPP from mevalonate pathway might be transfered from the cytoplasm to the plastids in the course of taxol and baccatin III biosynthesis. It was also presumed that different IPP biosynthesis pathways occur during different growth phases in *Taxus* cells [53].

Because of diterpenoid origin of paclitaxel, the special attention was paid to the investigation of MEP pathway regulation and cloning the appropriate genes. In general, 15 consequent secondary enzymatic reactions should be accomplished to form baccatin III– the key precursor of paclitaxel [54] (Fig. 2). Recent reviews reported cloning and characterization of 10 genes of taxane biosynthesis [43, 51 and references cited therein, 54, 55]. In particular, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase gene, which is the 5-th enzyme of the MEP pathway, was cloned from *T. medium* [56].

The efficiency of *Agrobacterium* transformation of yew cells is low and successful transformation protocol of *Taxus* cell suspensions was developed not long ago [57]. Because of this the majority of cloned genes were functionally expressed in *E. coli* и *Saccharomyces cerevisiae* [54, 58]. In the last case, 5 genes coding for 5 consequent reaction enzymes from primary metabolism to the intermediate taxadien-5-α-acetoxy-10-β-ol were installed in a single yeast host. It was shown that enzymes encoded by introduced heterologous genes utilized yeast isoprenoid precursors. However biosynthesis was blocked at the first cytochrome P450 hydroxylation step [54]. In order to enhance the hydroxylation activity, coexpression of cytochrome P450 reductase with cytochrome P450 oxygenase was successfully performed in yeast cells [59].

Among plant species, *A. thaliana* was transformed with recombinant *T. baccata* taxadiene synthase gene coding for plastid localized enzyme of one of early stages of paclitaxel biosynthesis catalyzing conversion of geranylgeranyl diphosphate to taxadiene. It led to accumulation of taxadiene in *Arabidopsis* cells [60]. This experiment demonstrated the perspective of approaches based on engaging of natural terpenoid precursors of plant host in taxane biosynthesis paythway. However, constitutive production of the full-length His-tagged enzyme in *A. thaliana* plants caused growth retardation and decreased the levels of photosynthetic pigments. Although these effects may be driven by a toxic taxadiene, the lower accumulation of endogenous plastid isoprenoids such as carotenoids and chlorophylls in transgenic plants also suggested the alteration of the balance of geranylgeranyl diphosphate pool. Using of inducible transgene expression system allowed optimization of taxadiene production which reached 30-fold higher levels than those in plants constitutively expressing the transgene [60]. Even higher taxadiene accumulation was observed after expression of taxadiene synthase in tomato fruits due to redirection of carotenoid metabolites: about 160 mg of taxadiene was extracted from 1 kg of freeze dried fruits [61].

Except for higher plants, taxadiene synthase was expressed in a moss *Physcomitrella patens* [62] and in the yeast *S. cerevisiae* [63]. Transgenic moss accumulated taxadiene up to 0.05 % of fresh weight. Transgene expression did not affect significantly the amounts of the endogenous diterpenoids. In contrast to other transgenic plants expressing heterologous taxadiene synthase, transgenic *P. patens* did not exhibit any growth inhibition due to the alteration of diterpenoid metabolic pools that suggests the perspective of this object for the biotechnological production of paclitaxel and its precursors.

Introduction of *T. chinensis* taxadiene synthase alone in *S. cerevisiae* did not increase the taxadiene levels because of insufficient levels of the universal diterpenoid precursor geranylgeranyl diphosphate. In order to attain a high level of taxadiene and its intermediate metabolites, geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius* and codon optimized *T. chinensis* taxadiene synthase gene were introduced into yeast genome. It resulted in 40-fold increase in taxadiene to app. 8.7 mg/L as well as significant amounts of geranylgeraniol (app. 33.1 mg/L), suggesting possibility for further increase of taxadiene level [63].

Scopolamine production. The anticholinergic tropane alkaloids hyosciamine, its racemic form atropine, and scopolamine have been known among the oldest drugs in the medicine because of their effect on parasympathetic nervous system. Currently they are widely used in pharmacology as muscle relaxants. These substances together with a

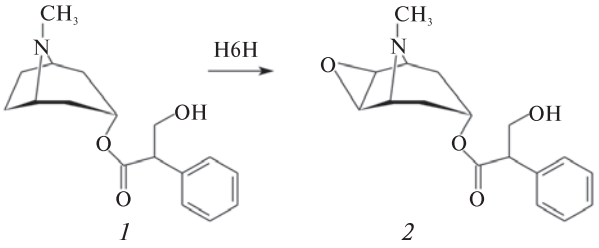

Fig. 3. Conversion of hyoscyamine (*1*) to scopolamine (*2*) by enzyme hyoscyamine 6β-hydroxylase (H6H)

number of other tropane alkaloids were isolated mainly from Solanaceae species, although tropane alkaloids were additionally detected in plants of several other families [64]. Hyoscyamine is normally the more abundant alkaloid in Solanacea species while scopolamine (which is more physiologically active and valuable) is produced in greater quantities only in *Duboisia* spp and *Datura metel* [64, 65]. As it was also shown for other natural products mentioned above, the chemical synthesis of these alkaloids has proved to be difficult and not economically feasible so that plant material is their only source. World demand for scopolamine was estimated to be about 10 times greater than that of hyoscyamine together with atropine [66]. This provoked the interest to tropane alkaloid biosynthesis pathway and biotechnological production of scopolamine. Because it was shown that undifferentiated systems such as calluses or cell cultures have low productivity [67], hairy roots caused by the infection of plants with *A. rhizogenes* have been chosen as an object for attempts to enhance scopolamine production. Owing to their stable and high productivity, hairy root cultures have been investigated for several decades for biotechnological production of the valuable metabolites (progress in understanding of secondary biosynthesis mechanisms in hairy root cultures was reflected in the recent reviews [68–71].

Hairy roots of *Hyoscyamus muticus* may produce high contents of hyoscyamine, but in many cases only trace amounts of scopolamine [72]. Sevon et al. described obtaining and analysis of hairy roots in more than 15 species. Amounts of scopolamine in the studied cultures varied from 0.2 to 32 mg/g DW. Laborious selection of the more productive clones and optimization of the growth conditions was often necessary to reach these levels of scopolamine accumulation [71]. Thus it is obvious that metabolic engineering of this biosynthetic pathway or its single steps could help to improve scopolamine production. In particular, the conversion of hyoscyamine to the much more valuable scopolamine could be regarded as the major goal of these studies.

Early stages of nicotine and tropane alkaloid biosynthesis are coinciding and discussed together with further reactions in the recent reviews [65, 73]. The first committed step of both pyridine and tropane alkaloid metabolism is *S*-adenosylmethionine (SAM)-dependent methylation of putrescine catalysed by putrescine *N*-methyltransferase, forming *N*-methylputrescine. The overexpression of *N. tabacum* putrescine *N*-methyltransferase (PMT) gene in scopolamine-rich *Duboisia* hybrids, *Datura metel, Atropa belladonna* and *H. muticus* caused increasing in accumulation of the direct metabolite N-methylputrescine (2–4-fold compared to wild type roots) [74], but there was no significant increase in either tropane or pyridine-type alkaloids [74–76] or the effect on the alkaloid level was only marginal. However, regulation of the expression of this gene can be crucial for alkaloid production in several species: in some transgenic *N. sylvestris* lines overexpression of *pmt* gene increased the nicotine content, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormal morphologies [75].

Scopolamine is 6,7β-epoxide derivative of hyoscyamine, formed from hyoscyamine in a two-step process via 6β-hydroxyhyoscyamine [78] by enzyme hyoscyamine 6β-hydroxylase (H6H) which can be classified as 2-oxoglutarate-dependent dioxygenase (Fig. 3). The enzyme was purified and characterized from *H. niger* [79]. The cDNA encoding *H. niger* H6H has been isolated by Matsuda et al. [80]. Additionally, H6H cDNA was cloned from several other scopolamine-producing Solanaceae species e.g. *A. baetica* [81], *A. belladonna* [82], *Anisodus tanguticus* [83] etc. Additionally, tropinone reductase, which catalyzes an earlier reaction of scopolamine biosynthesis in *H. niger*, has been cloned [84]. H6H gene from *H. niger* was placed under the control of 35S promoter and introduced to *A. belladonna* using *A. rhizogenes*. The obtained hairy roots contained up to five-fold higher concentrations of scopolamine than wild-type cultures [85]. Hyoscyamine was almost completely

converted to scopolamine in the leaves of transgenic *A. belladonna* plants expressing *h6h* gene. The level of scopolamine in the leaves reached up to 1.2 % DW [86]. Later, 35S-*h6h* gene was introduced into *H. muticus* producing high amounts of tropane alkaloids (up to 6 % of the dry weight in the leaves of mature plant). The best selected transgenic line produced 17 mg/L scopolamine, although conversion of hyoscyamine to scopolamine was still incomplete. In these examples overexpression of a single gene in the pathway has often led to an improved accumulation of the more valuable end product.

Further experiments included simultaneous overexpression of genes encoding PMT and the downstream H6H in *H. niger* hairy root cultures. It resulted in accumulation of significantly higher amounts of scopolamine (up to 411 mg/L,) in hairy root lines expressing both *pmt* and *h6h* genes compared with the control cultures (app. nine times more than that in the wild type) and transgenic lines harboring only one of the mentioned genes (more than two times higher level of scopolamine as compared with the best single-gene transgenic lines) [87].

Biotransformation was reported to be an alternative way for scopolamine production using non-hyoscyamine-producing transgenic systems fed with precursor hyoscyamine. Hairy roots of *N. tabacum* transformed with 35S-*h6h* gene have been studied for the production of scopolamine and nicotine alkaloids after feeding the cultures with hyoscyamine. In the optimal conditions the most productive clones of *N. tabacum* hairy roots converted up to 45 % of exogenous hyoscyamine to scopolamine; up to 85 % of the total scopolamine was released to the culture medium [88]. Recently, the protocol for bioconversion of hyoscyamine into scopolamine in bioreactor with *N. tabacum* cell suspension cultures was reported [89]. Functionally active H6H was obtained after heterologous expression of *h6h* gene from *Brugmansia candida* in *S. cerevisiae* [90].

Conclusions and future perspectives. In conclusion, cloning and heterologous overexpression of genes coding for several key enzymes of secondary metabolism often allowed considerable increasing of the level of valuable end product. The next step on the way to obtaining the commercial amounts of metabolite included correct choice of expression system and adaptation of the process to bioreactor scale. However, the efficient control of desired product synthesis requires a complete knowledge of all the steps in biosynthetic pathway, regulation mechanisms and cloning of the respective genes. It is difficult to forecast the results of introduction into plant genome of a single or reduced number of genes. Their overexpression may cause appearance of multiple rate-limiting steps and did not enhance production of desirable metabolite. It is necessary to consider the processes involved in the regulation of the whole pathway and interconnecting cellular pathways. Alternatively, translocation of gene cluster encoding the enzymes responsible for sequence of biochemical conversation in non-plant expression system can result in creation of highly efficient productive complex.

Ю.В. Шелудько

СОВРЕМЕННЫЕ ДОСТИЖЕНИЯ БИОТЕХНОЛОГИИ И ГЕНЕТИЧЕСКОЙ ИНЖЕНЕРИИ РАСТЕНИЙ ДЛЯ ПОЛУЧЕНИЯ ВТОРИЧНЫХ МЕТАБОЛИТОВ

С давних времен растения использовались людьми не только как пищевые культуры, но и для получения разнообразных химических соединений. Несмотря на современное развитие химических методов синтеза и микробиологических биотехнологий, растения остаются важнейшим и незаменимым источником биологически активных веществ. В обзоре мы сопоставили возможности и перспективы использования современных методов генетической инженерии в фармацевтической биотехнологии и привели примеры новейших систем, используемых для получения некоторых ценных натуральных продуктов – артемизинина, паклитаксела и скополамина.

Ю.В. Шелудько

СУЧАСНІ ДОСЯГНЕННЯ БІОТЕХНОЛОГІЇ ТА ГЕНЕТИЧНОЇ ІНЖЕНЕРІЇ РОСЛИН ДЛЯ ОТРИМАННЯ ВТОРИННИХ МЕТАБОЛІТІВ

З давніх часів люди використовували рослини не тільки як харчові культури, але і для отримання різноманітних хімічних сполук. Незважаючи на сучасний розвиток методів хімічного синтезу й мікробіологічних біотехнологій, рослини залишаються найважливішим і незамінним джерелом біологічно активних речовин. В огляді ми зіставили можливості й перспективи використання сучасних методів генетичної інжене-

рії в фармацевтичній біотехнології і навели приклади сучасних біотехнологічних систем, які застосовують для одержання деяких цінних натуральних продуктів – артемізініна, паклітаксела і скополаміна.

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