

Gene Engineering of Lamiaceae Family: Basic and Applied Research

Mikhail Yur. Cherednichenko¹; Oksana B. Polivanova²; Daria An. Khlebnikova³; Anastasiya Vl. Sosina⁴; Mariia M. Belova⁵; Elena An. Kalashnikova⁶

¹Candidate of Biological Sciences, Associate Professor, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia.

¹michael.tsch@gmail.com

²Candidate of Biological Sciences, Senior Lecturer, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia.

²polivanovaoks@gmail.com

³PhD Student, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia.

³libelle.91@gmail.com

⁴PhD Student, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia.

⁴sosina_2012@mail.ru

⁵MSc Student, Moscow Institute of Physics and Technology (National Research University), Russia.

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Russia.

⁵belova.pavla2015@yandex.ru

⁶Doctor of Biological Sciences, Professor, Head of Department, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia.

⁶kalash0407@mail.ru

Abstract

Gene engineering is a convenient way of solving basic and applied tasks related to the system of secondary metabolism of medicinal and aromatic plants, such as a large and economically important plant family Lamiaceae Martinov (Labiatae Juss.) that includes such major species, as Agastache, Dracocephalum, Lavandula, Mentha, Ocimum, Salvia, Satureja, Thymus, etc. The basic value of this method consists in studying the genetic mechanism of secondary metabolism, which includes key enzyme genes, transcription factors, etc. Applied research consists in producing valuable secondary metabolites with biological activity, including active pharmaceutical ingredients in both, the family's plants proper and the heterologous systems using relevant plant genes. This overview considers a set of diverse gene engineering studies.

Key-words: Lamiaceae, Medicinal and Aromatic Plants, Biotechnology, Gene Engineering, Metabolic Engineering, *In Vitro*, Hairy Roots, Secondary Metabolism.

1. Introduction

Medicinal and aromatic plants are a bank of valuable substances of secondary synthesis (secondary metabolites, SM) a lot of which exhibit a defined biological activity. A major niche in the production of bioactive substances belongs to the family Lamiaceae Martinov (Labiatae Juss.), including such plants, as giant hyssop, dragonhead, lavender, mint, basil, sage, savory, etc. They contain all main classes of SM, first of all, terpenoids and phenolic compounds (PC) but alkaloids as well. The natural populations of Lamiaceae representatives shrink in number, and many species are endangered (<https://www.iucnredlist.org/>). Biotechnology allows preserving the available biodiversity of medicinal and aromatic plants, studying the patterns of accumulation of valuable SM, and cultivating highly productive plant lines in artificial conditions. Plant biotechnology methods can be divided in two main groups, including tissue and cell culture (cell engineering) and gene engineering. Genome editing often separated from and even opposed to gene engineering is formally included in it. The recently invented term metabolic engineering is closer to the notion of gene engineering, though metabolic changes can be made by cell engineering techniques as well. Gene engineering that allows activating and deactivating genes, regulating their expression, and transferring genes from other objects of the same or a different kingdom allows solving a number of basic and applied tasks.

Gene Engineering as a Method of Modifying Plant Resources

The three decades of developing the gene modification of agricultural and decorative plants have witnessed a significant progress in terms of improving their agronomic traits, such as resistance to herbicides, fungal, bacterial, and viral diseases, insects, unfavorable abiotic factors, as well as the quality of reserve proteins of cereals, fatty acid composition of oil crops, decorativeness, etc. At the same time, genetic transformation techniques are involved in gradual evolution and the systems become more and more accurate (precision) and thus gradually solve the problem of the undirected insertion or change in DNA sequences in the genome.

Thus, a survey by a group of authors from China is about the genetic transformation mechanism using the *Agrobacterium rhizogenes* Ri-plasmid, with the production of *hairy roots* as an efficient system of producing valuable SM, as well as about the current research and lines for future research, and thus proposes a new approach to studying SM of *hairy roots* at the level of gene expression (Liu et al., 2015). In the opinion of the Russian scientists from the Central Botanic Garden of the Siberian Branch of the Russian Academy of Sciences (SB RAS) and the National Research

University — Tomsk State University, this system of cultivation can be used more efficiently by engaging proteomic data for identifying (de-)differentiation key proteins to control these processes. The supplementation with metabolome and molecular biological data on the basis of using mathematical tools must result in building metabolic models of medicinal plants, which will expand the potential of biotechnology (Erst et al., 2016).

A DNA modification technique considered as promising in the surveys by Russian scientists from the Institute of Cytology and Genetics of SB RAS is genome editing. The authors compare various techniques of optimizing the CRISPR/Cas9 system that can expand its capabilities: *in silico* selection of target sites, vector design, vector transfer techniques, transfer efficiency and expression estimation, etc. (Khlestkina and Shumnyi, 2016; Gerasimova et al., 2017). Another survey of this team analyzes the use of the CRISPR/Cas9 modification of agricultural plants. The sources found by the authors describe 145 target genes in 15 agricultural plants (in particular, the highest number of 78 modified genes was found for rice). According to the survey's authors, editing is most often basic and less than a half of the works are about the applied editing for improving agronomic traits; they agitate the academic community for expanding the application of this technology to obtain more information, including the one about the restrictions of this technique (Korotkova et al., 2017).

K. E. French, a scientist from the United States, has described a new transformation technique based on carbon nanoparticles; this technique can be used for transforming bacteria, plants, and algae (French, 2019).

The work by a group of Spanish authors is about developing the system of genetically transforming plants with antifungal proteins on the basis of tobacco mosaic virus (TMV). Recombinant proteins are produced after the inoculation of plant leaves with the formation of infection clones. The authors use this simple vector to generate two different antifungal proteins in the leaves of *Nicotiana benthamiana* against *Aspergillus giganteus* and *Penicillium digitatum*. The high output of protein was achieved by directing them to apoplast (when they were directed to intracellular compartments, toxic effect were observed and the protein level sharply decreased to undefined amounts) (Shi et al., 2019).

Studying Structure and Functions of DNA Sequences

The basic value of gene engineering consists in studying the structure and functions of DNA sequences of three plant cell genomes (of nucleus, chloroplasts, and mitochondria).

The survey by scientists from the United States and Taiwan also considers the influence of the knowledge of chloroplast genome sequences on understanding the origin of economically important cultivated plant species and the changes that occurred in the course of breeding, and also on the potential opportunities for applying biotechnology techniques. The interest in using chloroplast genomes in practice is encouraged by the availability of more than 800 sequenced genomes of chloroplasts of various terrestrial plants, which has already expanded our understanding of the biology of chloroplasts, intracellular gene flow, biodiversity preservation and genetic basics with the help of which chloroplast transgenes can be transformed for improvement of agronomic traits of plants or for producing valuable agricultural or biomedical products (Daniell et al., 2016).

For example, a survey by authors from Hungary and India presents plastid engineering techniques and the opportunities for applying them to improve the quality and productivity of farming crops in unfavorable growth conditions - transplastom plants: they produce insecticidal and antimicrobial compounds (resistant to pests and requiring less pesticides), resist abiotic stress factors (including the application as phytoremediants), are more productive as a result of improved photosynthesis, and have an elevated content of mineral substances (Wani et al., 2015).

A group of authors from Japan have studied mitochondrial genes responsible for cytoplasmic male sterility (CMS). In the work they have eliminated the CMS-associated genes (*orf79* and *orf125*) of CMS varieties of rice and rape, respectively, using Transcription Activator-Like Effector Nucleases (TALEN) with signals of localization in mitochondria (mitoTALEN). The authors have shown that the knockout of these genes restores male fertility and thus confirmed that these genes are the ones that cause CMS. As shown by the sequencing, mitoTALEN-induced two-chain breaks were restored by homologous recombination and that these target genes and the surrounding sequences were removed in the course of the process. Thus the authors have proved that mitoTALEN can be used in the stable and heritable modification of the mitochondrial genome in plants (Kazama et al., 2019).

An international group of scientists from Australia, China, and the United States have modified the CRISPR/Cas9 for improving the output of mutants in T₁ and T₂ generations on the model object *Arabidopsis thaliana*. Usually, constitutive promoters result in the low efficiency of heritable mutations in T₁ generation: for example, the expression of Cas9 under the CaMV 35S-promoter caused 2.3 % of mutations in the T₁ plants and did not cause any homozygotic mutations in T₁ and T₂ generations. The using of YAO and CDC45 as two promoters specific to cell division resulted in a mutation frequency of 80.9 to 100 % in T₁ generation, with non-chimeric

mutations in generations T₁ (4.4...10 %) and T₂ (32.5...46.1%). The authors also replaced constitutive ubiquitin promoter with promoter CDC45 in the previously described CRISPR/Cas9 multiplex system.

The modified system caused a higher mutation frequency in T₁ generation than the original system (60.17 % and 43.71 %, respectively) and also resulted in more efficient heritable mutations (11.30 % and 4.31 %, respectively) (Feng et al., 2018).

A group of authors from the United States point at the difficulties of regenerating plants with an edited genome (for example, with the help of CRISPR/Cas9) using a tissue culture and consider two methods of creating edited dicotyledons by the *de novo* induction of meristem formation. Development regulators and gene-editing agents are supplied to somatic plant cells, which causes the origination of meristems that produce shoots with target DNA modifications, which are passed on the next generation. The *de novo* induction of meristem formation avoids complexities in tissue culture and holds out the prospect of overcoming the bottleneck in editing plant genes (Maher et al., 2019).

The work by another group of scientists from the United States studied the structure and epigenome status of T-DNA insertion in the course of *Agrobacterium*-mediated transformation. The authors analyzed the *Arabidopsis thaliana* lines from three broadly used collections of T-DNA inserts (SALK, SAIL, and WISC). The *de novo* assembly based on Nanopore sequencing for two segregating lines has partially explained T-DNA structures and detected multiple translocations and exchanges of chromosomal arm ends. The resulting contigs have allowed correcting 83 % of non-centromeric misassemblies in the current reference genome TAIR10. The continuous resolution at the nucleotide level has allowed conducting an in-depth study of epigenome in the sites of T-DNA inserts. The insertions of line SALK_059379 were rich in 24-nucleotid small interfering RNA

(siRNA) and the dense methylation of DNA cytosine, which suppressed the expression of the transgene. The insertions of T-DNA into the line SAIL_232 are mainly the targets of 21/22-nucleotid siRNA, in which case the methylation and silencing of DNA are conditioned by the reporter, not by the resistance gene. In addition, the authors have profiled the H3K4me3, H3K27me3, and H2A.Z chromatin environment of the T-DNA insertions using ChIP-Seq in SALK_059379, SAIL_232, and five extra T-DNA lines and detected various effects, from the complete loss of chromatin cells to the *de novo* inclusion of H2A.Z and trimethylation of H3K4 and H3K27 around the sites of the T-DNA insertion. This study allows having a new look on the structural influence of the intrusion of foreign fragments in plant genomes (Jupe et al., 2019).

The visualization of the spatiotemporal genome organization improves the understanding of the interrelation of the structure and function of chromatin. A group of authors from Germany and Japan has developed a tool for visualizing certain genome sequences in fixed nuclei and chromosomes on the basis of two-part guide RNA with the recombinant complex of the endonuclease Cas9. This method requires no special transformation construction or technique. Unlike classical fluorescent *in situ* hybridization, RGEN-ISL (RNA-guided endonuclease – *in situ* labelling) does not require denaturing DNA and, therefore, allows better preserving the chromatin structure. Differentially marked trans-activating crRNAs allow creating the RGEN-ISL multiplex. In addition, this procedure is compatible with immunohistochemistry. The kinetics of this reaction has been detected by the real time visualization of CRISPR/Cas9-mediated DNA labelling. The broad range of RGEN-ISL adaption to various temperatures and combinations of techniques, does have the potential for developing the chromosome biology (Ishii et al., 2019).

Expression of Secondary Metabolism Genes in Heterologous Systems

An important topic of practical gene engineering is the expression of genes in heterologous systems. For example, a survey by authors from China and the United States considers cases of applying the heterologous system of biosynthesis for such important classes of natural compounds as terpenoids, flavonoids, alkaloids, and polyketides. The authors present tools and strategies at various levels, including genes, metabolic pathways, genomes, and populations (Luo et al., 2015). A survey by scientists from Greece and the United States presents the information about rationalized approaches to producing natural or artificial flavonoids with the help of biotechnology; these approaches analyze the value of combinatorial biosynthesis of agricultural/pharmaceutical compounds produced in heterologous organisms. The authors also consider the strategies and achievements of synthetic biology with the focus on metabolic engineering aimed at modifying flavonoid-producing microorganisms and plants. Some attention is also paid to the rapidly growing number of completed genomes the information on which favours the identification of genes responsible for the accumulation of species-specific SM (Trantas et al., 2015).

A survey by scientists from Denmark and Malaysia is devoted to prospects of the biotechnological production of valuable terpenoids in heterologous systems. A lot of terpenoids have a fairly complicated structure, which is why full chemical synthesis often requires many phases and complex chemical reactions, which leads to a low substance output or abnormal stereochemistry. This issue can be resolved by using the strategy of obtaining a higher product output or expression of

biosynthesis pathways in heterologous hosts. There are cases of the successful construction of microorganisms, such as yeast, fungi and bacteria, for producing such compounds; it usually requires making significant changes in the metabolism of the host organism because the process is inhibited by the need for optimizing the expression of plant genes, post-translation protein modifications, subcellular localization control, etc. This is why, the authors present metabolic plant engineering as an efficient method of producing valuable natural compounds of plant origin (Ikram et al., 2015). The survey by S.E. O'Connor from The John Innes Centre (UK) considers engineering approaches to secondary metabolism in both, heterologous and native hosts. It emphasizes the growing importance of compartmentalization in metabolic engineering. In addition, the engineering approaches to changing the structure of key SM classes are subjected to a critical appraisal (O'Connor, 2015).

A work by a team of Danish and Swedish scientists describes the production of transgenic single-cell cyanobacteria *Synechocystis* for the expression of 13R-malolil oxide (13R-MO) – the precursor for forskolin, a valuable diterpenoid accumulated in *Coleus forskohlii* plants (Lamiaceae) hard and expensive to produce by chemical methods. The added enzyme genes from the plant ultimately allowed producing up to 0.45 mg 13R-MO/g DW (Englund et al., 2015).

A team of scientists from Denmark has created the transgenic yeast strain for producing *p*-coumaric acid, an intermediate compound for synthesizing aromatic SM. That strain accumulated 1.93 ± 0.26 g/l *p*-coumaric acid in the course of fermentation with the periodic substrate addition. Then the authors analyzed the strain and showed that it had transcriptional abnormalities in the genes involved in transporting amino acids and sugars, which could be the response against the stress caused by the production of *p*-coumaric acid. The authors have also developed the yeast strain expressing the flavonoid metabolic pathways and containing up to ten heterologous genes. They could synthesize six different types of flavonoids (for example, kaempferol and meletin). Those scientists were the first to synthesize with the help of yeast such flavonoids, as liquitrigenine, resokaempferol, and fisetin (Rodriguez Prado et al., 2016).

A team of scientists from Portugal and the United States have created the transgenic *Escherichia coli* strain able to synthesize caffeic acid from tyrosine as a precursor. The structure of this pathway included tyrosine ammonia lyase (TAL) from *Rhodotorulaglutinis* (yeast) for transforming tyrosine to *p*-coumaric acid and 4-coumarate-3-hydroxylase (C3H) from *Saccharothrixspanaensis* (bacterium) or cytochrome P450 CYP199A2 from *Rhodopseudomonaspalustris* (bacterium) for transforming *p*-coumaric acid to caffeic acid. The genes were codon optimized; various combinations of plasmids were used to increase the titer of caffeic

acid. The highest caffeic acid output produced using TAL and CYP199A2 as well as TAL and C3H was 1.56 mM (280 mg/l) and 1 mM (180 mg/l), respectively. In the opinion of the authors this study provides prerequisites for further developing the synthesis of more complex SM, such as flavonoids and curcuminoids (Rodrigues et al., 2015).

A work by Chinese authors presents the heterologous expressing genes of benzyl isothiocyanate biosynthesis in *Escherichia coli*. The isothiocyanates contained in large amounts in the Brassicaceae representatives reduce the risk of some kinds of cancer and cardiovascular diseases. The authors constructed two chimeric enzymes of cytochrome P450 and functionally expressed in *E. coli*. The C-S-liase of plant origin was replaced with cystathionine-B-lyase of *E. coli*. In addition, the desulphoglucosinolate: PAPS-SULT from *Arabidopsis thaliana* (Col-0 ecotype) and the myrosinase from *Brevicoryne brassicae* (aphid) were used. The biosynthesis of benzyl isothiocyanates by the combined expression of *in vitro* optimized enzymes was confirmed by GC-MS analysis (Liu et al., 2016).

The possibilities of studying and modifying the metabolite composition of plants have also been and remain the object of research conducted by gene engineers.

Since isoprenoids in plants are synthesized from precursors along two pathways separated in space (cytoplasmic/peroxisomal mevalonate pathway (MVA) and plastid methyl erythritol phosphate pathway (MEP)), American scientists have created transgenic lines of the model plant *Arabidopsis thaliana* with a changed expression level of each separate gene engaged in both metabolic pathways.

While evaluating the correlation of the transgene expression levels with the metabolites of isoprenoid markers (gene-metabolite correlation), the authors defined the relative importance of the transcription control in each separate phase of precursor biosynthesis for isoprenoids. The accumulation model for intermediate metabolite (gene-metabolite correlation) was later used for defining bottlenecks in the sterol metabolic pathway. The degree of metabolic cross coupling and exchange of isoprenoid intermediates among the pathways separated in space was evaluated with the help of the combination of gene-metabolite and metabolite-metabolite correlation analyses. This strategy allows selecting genes for metabolic engineering. The overexpression of defined gene combinations can be used for significantly increasing the accumulated amount of sterols, which will positively affect the biomass of agricultural and energy plants (Lange et al., 2015).

A team of British scientists has created the transgenic hexaploid wheat with the gene of farnesyl diphosphate - the precursor for (E)- β -farnesin (E β f), the pheromone alarm signal for harmful

plant aphids. The lab tests revealed a noticeable repellent effect, but it was not confirmed under field conditions (Bruce et al., 2015).

The representatives of Lamiaceae family have been exposed to genetic modification relatively rarer than many other agricultural plants; however, these studies have a huge potential; like with other plants, it includes both values, basic and applied. The basic value consists, first of all, in studying the mechanism of secondary metabolism, and the applied value consists in controlling this metabolism (Floryanowicz-Czekalska, Wysokińska, 2000).

Hairy Roots Culture

A large number of gene engineering studies of aromatic and medicinal Lamiaceae plants have to do with creating the *hairy roots* culture by transforming the plants with strains of *Agrobacterium rhizogenes* with various *rol*-genes.

For example, scientists from Iran have induced the formation of *hairy roots* in the medicinal plant *Perovskiaabrotanoides*, by using various strains of *A. rhizogenes* (ATCC15834, TR105, and R1000), and studied the influence of acetosyringone and carbon sources on the probability of rooting and root biomass formation. The potential of *hairy roots* was studied relatively to the tanshinone synthesis. The highest transformation frequency was observed in the nodes infected with ATCC15834 (47.33 %), and it grew to 60.99 % with the addition of 100 μ M of acetosyringone. The PCR analysis using the *rolC*-specific primer with an amplicon of 612 bp confirmed the transgenic nature of *hairy roots*. Finally, as shown by HPLC analysis, the cryptotanshinone and IIA tanshinone content in *hairy roots* (after the transformation with strain TR105) was 53.17 ± 0.26 , and $14.48 \pm 0,30$ μ g/g DW, respectively (Ebrahimi et al., 2017).

A team of Brazilian and Canadian scientists has produced *hairy roots* of *Origanum vulgare* plants using *A. rhizogenes* (strains K599 and ATCC15834), as well as developed a plant regeneration protocol. After the infection the *hairy roots* formation frequency varied depending on the concentration of nutrient medium components. A high transformation frequency was observed on the modified MS nutrient medium (91.3 %). On the medium MS + 0.25 mg/l 2,4-dichlorophenoxyacetic acid the induction frequency of callus genesis on explants from *hairy roots* was 81.5 %.

The highest frequency of shoot regeneration (85.18 %) and rhizogenesis was observed on the media MS + 0.25 mg/l 6-benzylaminopurine and MS + 0.25 mg/l indole-3-butyric acid (Habibi et al., 2016).

The Iranian scientists produced *hairy roots* of *Agastache foeniculum* with the help of *A. rhizogenes* (strains A4, A7, and 9435) on various explant types (from hypocotyl, cotyledon, and leaf). The highest transformation frequency was achieved with the influence of the A4 strain on the leaf explants obtained from single-month leaves (51.1 %). The transgenic nature of *hairy roots* lines was confirmed by PCR analysis. As shown by HPLC, the synthesis of SA in the transformed roots of *A. foeniculum* was about fourfold as high as in non transformed. A separate experiment was conducted in which the obtained *hairy roots* were cultivated in a liquid medium with the addition of various concentrations of SA (0 to 1 mM), chitosan (0...150 mg/l) (as an elicitor), and sucrose (20...50 g/l). The results showed that the addition of sucrose and chitosan in concentrations of 30 g/l and 100 mg/l increased the biomass of *hairy roots*, whereas, on the contrary, SA inhibited their growth (Nourozi et al., 2014). In their subsequent experiments the same authors checked the efficiency of the influence of lighting and darkening conditions as well as two inoculation techniques (immersion and injection); in the immersion technique the influence of the inoculation period (3, 5, and 7 mins) on the *hairy roots* induction was studied. In addition, the influence of various nutrient media (MS, ½ MS, and B5) on the growth of *hairy roots* was studied. The RA content in the transformed and untransformed roots was analyzed by HPLC. The several inoculation techniques turned out to have no major differences in terms of the *hairy roots* induction ability; in case of immersion, however, a better efficiency was reached at a 5-minute exposure. Good lighting was more preferable for inducing *hairy roots* than darkening. The maximal *hairy roots* growth rate was attained on the ½ MS nutrient medium. The RA content in transformed roots (213.42 µg/g DW) was much higher than in non transformed roots (52.28 µg/g DW) (Nourozi et al., 2016).

Scientists from Iran have developed an efficient system of inducing the *hairy roots* formation for *Dracocephalum kotschyi*. Five strains of *A. rhizogenes* were used, including A4, ATCC15834, LBA9402, MSU440, and A13 (MAFF-02-10266). A sharp increase in the transformation frequency was observed on the modified MS medium without macro salts. The maximal frequency of the *hairy roots* induction on that medium was observed after the transformation with strains MSU440 (89 %) and ATCC15834 (80 %). The highest number of shoots and their maximal regeneration frequency were registered later on the medium MS + 0.25 mg/l BAP + 0.1 mg/l of 1-naphthalene-acetic acid. Then the medium MS + 0.5 mg/l IBA was used for rooting the obtained shoots (Sharafi et al., 2014).

Polish scientists grew *hairy roots* of a different dragonhead species – *Dracocephalum moldavica* – using the A4 strain of *A. rhizogenes*. The transformed roots were produced from explants with a low transformation frequency (up to 3 %). *Hairy roots* were cultivated by the photoperiod

(16 hours daylight) and in the darkness. The largest biomass of *hairy roots* (7.23 g FW or 0.89 g DW per vessel) was obtained for the plants cultivated on the Woody Plant Medium by the periodic lighting. HPLC analysis showed the highest RA content (78 mg/g DW) in the roots cultivated on the B5 medium by the photoperiod; that content was about 10 times as high as in the roots of parent field plants. The methanolic extract of *hairy roots*, grown on the B5 medium by the photoperiod, displayed the highest antioxidant activity, much higher than the methanolic extract of field-grown plants. The most active methanolic extract of *hairy roots* was characterized by the highest RA content and total PC content (Weremczuk-Jeżyna et al., 2013).

Studying Genetic Mechanism of Secondary Metabolism

Some part of gene engineering works consists in identifying the mechanism of secondary metabolism, first of all, with the identification of genes of metabolic pathway enzymes and possibilities of regulation their expression.

Korean scientists studied the molecular structure and regulation of the flavonoid biosynthesis in *Scutellaria lateriflora*. Five cDNA were isolated that coded the enzymes involved in the biosynthetic pathway of flavonoids; the enzymes were phenylalanine-ammonialyase (SIPAL), cinnamate-4-hydroxylase (SIC4H), 4-coumarate-CoA-lygase (SI4CL), chalcone synthase (SICHs) and chalcone isomerase (SICHI). As shown by the gene expression analysis, they were expressed at a high level in all of the studied organs by the highest wogonin content in the roots. Baikalin and baikalein accumulated in the plants in different ways, and the highest concentration of baikalin and baikalein was registered in the leaves and stems, respectively. The exogenous MeJa significantly intensified the expression of SICHs and SICHI and the accumulation of baikalin (22.54 mg/g), baikalein (1.24 mg/g) and wogonin (5.39 mg/g) in *hairy roots* (the accumulation increased by 644, 138, and 112 % respectively).

The expression level of SICHs, the first phase of the flavonoid biosynthesis in *hairy roots* strengthened after 3 and 4 weeks of development in lighting conditions. *Hairy roots*, grown in darkness, had a higher baikalin and baikalein content than the *hairy roots* grown in lighting conditions; however, the latter accumulated more wogonin (Tuan et al., 2018).

The recent years have witnessed an upsurge in the popularity and application of the plant genome editing with the help of the CRISPR/Cas9 system that implies manipulations with the genetic material available in the genome. For example, Chinese scientists used that system to knock out the gene of diterpene synthase (SmCPS1), the enzyme of tanshinone biosynthesis, in *Salvia miltiorrhiza*.

Three homozygotic and eight chimeric mutants were obtained from 26 independent transgene lines of *hairy roots* by the transformation with *A. rhizogenes*. According to metabolome analysis (LC-qTOF-MS and Q-TRAP-LC-MS/MS), homozygotic mutants have no tanshinones at all whereas in chimeric mutants their content is low (Li et al., 2017).

Scientists from Singapore and the United States studied the TF regulating secondary metabolism in peltate glandular trichomes (PGT) of *Mentha spicata*.

The authors separated and functionally characterized the new gene MsYABBY5, that is active mainly in the PGT of mint. The authors have created various lines of transgenic plants, where MsYABBY5 was either too expressed or suppressed with the help of RNA interference. According to the analysis of those lines, the suppression of the MsYABBY5 expression resulted in an elevated terpene content whereas overexpression decreased their content. In addition, the ectopic expression of MsYABBY5 in *Ocimum basilicum* and *Nicotiana sylvestris* reduced the SM synthesis, which allowed assuming that the coded TF was probably the repressor of secondary metabolism (Wang et al., 2016).

Applied Research

First of all, the applied research in genetic modification have to do with attempts of increasing the synthesis of valuable SM.

A work by scientists from Croatia is about the genetic elicitation of the PC accumulation in *Plectranthus scutellarioides* (syn. *Coleus blumei*). The protein elicitor, β -cryptogein from oomycetes, can result in a hypersensitive response and acquired systemic resistance to some pathogens, which is due to an increased the PC accumulation. The respective gene was put under the control of an alcohol-induced promoter and transferred to coleus by agrobacterial transformation. The expression of β -cryptogein in *hairy roots* was controlled by using 1 % and 2 % ethanol during the 21-day induction period. The expression significantly decreased the content of soluble PC and RA in the *hairy roots* lines but, at the same time, increased the secretion of PC, RA, and caffeic acid into the culture medium (Vuković et al., 2013).

A group of authors from the United States genetically modified *Mentha x piperitoides* to evaluate the capabilities of metabolic engineering for improving the output and composition of EO. Its output was increased through the excessive expression of the genes involved in supplying the precursors along the

MEP-pathway. In addition, the combinations of two genes were evaluated for the change in both, oil output and composition in one transgenic line. The most promising results were achieved by

transgenic plants that expressed the antisense version of (+)-menthofuran synthase RNA for reducing the accumulation of methofuran. The expression of the gene coding (+)-limonene synthase was used to accumulate an increased amount of (+)-limonene (Lange et al., 2011).

In 1999 a group of French scientists developed the protocol for the genetic transformation of lavandin (*Lavandula × intermedia*). This aromatic plant accumulates valuable EO actively used in perfumery and cosmetic and pharmaceutical industries. The test involved checking five agrobacterial strains. The transformed callus lines were obtained using strains AGL1/GI, EHA105/GI, and C58/GI. The vector structures carried the resistance gene to kanamycin and the gene of β -glucuronidase. The presence of an insert was confirmed by PCR analysis and Southern blot hybridization. The expression was confirmed by a β -glucuronidase test as well as by RT-PCR. The transformation efficiency ranged from 3 to 9 %, depending on the strain (Dronne et al., 1999).

The main components of the EO in spike lavender (*Lavandula latifolia*) are monoterpenes produced from the precursors isopentenyl diphosphate and dimethyl allylphosphate through the plastid MEP-pathway and/or cytoplasmic MVA-pathway. 1-desoxy-D-xylulose-5-P-synthase (DXS) catalyses the first phase of the MVA-pathway. A group of scientists from Spain expressed the cDNA, coding DXS from *Arabidopsis thaliana*, in spike lavender. According to the GC-MS analysis, the transgenic plants accumulated much more EO as compared with the control plants (by 250 and 500 % higher in the leaves and flowers, respectively). Transgenic T₀ plants were grown for 2 years, exposed to mandatory self-fertilization, and thus the seeds of T₁ were obtained. The type of DXS-transgene inheritance in the T₁ generation was studied. The phenotype with an elevated EO content was preserved in the posterity (Muñoz-Bertomeu et al., 2006). In 2007 the same authors created the transgenic spike lavender that expressed the cDNA of *Arabidopsis thaliana* HMG1, coding the catalytic domain of 3-hydroxy-3-methyl glutaryl-CoA-reductase (HMGR1S), the key enzyme of the MVA-pathway. Transgenic T₀ plants accumulated much more components of EO as compared with the control plants (by up to 110 and 80 % more in leaves and flowers, respectively). The overexpression of HMGR1S also increased the amount of such products, as sterols, β -sitosterol, and stigmasterol (by 80 and 90 %, respectively), but had no effect on the accumulation of carotenoids and chlorophylls. The authors analyzed the T₁ plants, obtained from the self-fertilized T₀ lines: the elevated levels of EO and sterols were still retained in the posterity (Muñoz-Bertomeu et al., 2007).

2. Conclusion

Thus the gene engineering modification technology, genome editing included, provides most ample opportunities to biotechnologists, who specialize in medicinal and aromatic plants, for improving the quality of plant raw materials, decorative effect of plants, and also the output of valuable secondary metabolites, In addition, gene engineering techniques allow understanding the mechanism of such a complex system, as secondary plant metabolism, which will undoubtedly favor the development of biotechnological production of cells, tissues, and whole Lamiaceaplants with a high potential for accumulating bioactive substances.

Appendices

Appendix. Key to the main abbreviations used in the paper

4CL	4-coumarate-CoA-lygase
B5	Gamborgnutrient medium
BAP	6-benzylaminopurine
C3H	4-coumarate-3-hydroxylase
C4H	cinnamate-4-hydroxylase
CHI	chalconeisomerase
CHS	chalconesynthase
CMS	cytoplasmatic male sterility
CPS	copalyldiphosphatesynthase
DW	dry weight
EO	essential oil
FW	freshweight
HPLC	high-performance liquid chromatography
IBA	indole-3-butyric acid
LC-qTOF-MS	liquid chromatography quadrupole time-of-flight with mass-spectrometry
MeJa	methyljasmonate
MS	Murashige and Skoognutrient medium
NAA	1-naphtaleneacetic acid
PAL	phenylalanine-ammonialyase
PC	phenolic compounds

Q-TRAP-LC-MS/MS	quadrupole ion trap liquid chromatography with tandem mass spectrometry
RA	rosmarinicacid
RGEN-ISL	RNA-guided endonuclease – <i>in situ</i> labelling
RT-PCR	reverse transcription polymerase chain reaction
SA	salicylicacid
SM	secondarymetabolites
TAL	tyrosine-ammonialyase
TALEN	Transcription Activator-Like Effector Nucleases
TF	transcriptionfactor

References

- Gerasimova, S.V., Khlestkina, E.K., Kochetov, A.V., & Shumnyi, V.K. (2017). Genome Editing System CRISPR/Cas9 and Peculiarities of Its Application in Monocots. *Russian Journal of Plant Physiology*, 64(2), 92-108.
- Korotkova, A.M., Gerasimova, S.V., Shumnyi, V.K., & Khlestkina, E.K. (2017). Crop Genes Modified Using CRISPR/Cas System. *Vavilov Journal of Genetics and Breeding*, 21(2), 250-258.
- Khlestkina, E.K. & Shumnyi, V.K. (2016). Prospects for Application of Breakthrough Technologies in Breeding: The CRISPR/Cas9 System for Plant Genome Editing. *Russian Journal of Genetics (Genetika)*, 52(7), 774-787.
- Erst, A.A., Zhelezniichenko, T.V., & Badulina, A.A. (2016). *Hairy Root Cultures as a Source of Biologically Active Substances of Medicinal Plants. European research*, 1(12), 41-43.
- Bruce T.J.A., Aradottir G.I., Smart L.E., Martin J.L., Caulfield J.C., Doherty A., Sparks C.A., Woodcock C.M., Birkett M.A., Napier J.A., Jones H.D., & Pickett J.A. (2015). The first crop plant genetically engineered to release an insect pheromone for defence. *Nature*, 5.
- Daniell, H., Lin, C.S., Yu, M., & Chang, W.J. (2016). Chloroplast genomes: diversity, evolution, and applications in genetic engineering. *Genome biology*, 17(1), 1-29.
- Dronne, S., Moja, S., Jullien, F., Berger, F., & Caissard, J.C. (1999). Agrobacterium-mediated transformation of lavandin (*Lavandula x intermedia* Emeric ex Loiseleur). *Transgenic research*, 8(5), 335-347.
- Ebrahimi, S., Zaker, A., Abrishamchi, P., Bahrami, A.R., Ganjeali, A., & Sodagar, N. (2017). Hairy root induction and secondary metabolite production in *Perovskia abrotanoides* Karel. *Journal of Plant Process and Function*, 6(20), 17-26.
- Englund, E., Andersen-Ranberg, J., Miao, R., Hamberger, B., & Lindberg, P. (2015). Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. *ACS synthetic biology*, 4(12), 1270-1278.
- Feng, Z., Zhang, Z., Hua, K., Gao, X., Mao, Y., Botella, J.R., & Zhu, J.K. (2018). A highly efficient cell division-specific CRISPR/Cas9 system generates homozygous mutants for multiple genes in *Arabidopsis*. *International journal of molecular sciences*, 19(12), 3925.

Floryanowicz-Czekalska, K., & Wysokińska, H. (2000). Transgenic shoots and plants as a source of natural phytochemical products. *Acta Societatis Botanicorum Poloniae*, 69(2), 131-136.

French K.E. (2019). *Nanoparticle-mediated transformation expands horizon of organism engineering*. bioRxiv. <https://doi.org/10.1101/559252>

Habibi, P., De Sa, M.F.G., Da Silva, A.L.L., Makhzoum, A., Da Luz Costa, J., Borghetti, I.A., & Soccol, C.R. (2016). Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*. *Physiology and Molecular Biology of Plants*, 22(2), 271-277.

Ikram, N.K.B.K., Zhan, X., Pan, X.W., King, B.C., & Simonsen, H.T. (2015). Stable heterologous expression of biologically active terpenoids in green plant cells. *Frontiers in plant science*, 6, 129.

Ishii, T., Schubert, V., Khosravi, S., Dreissig, S., Metje–Sprink, J., Sprink, T., & Houben, A. (2019). RNA- guided endonuclease–in situ labelling (RGEN-ISL): a fast CRISPR/Cas9- based method to label genomic sequences in various species. *The New Phytologist*, 222(3), 1652-1661.

<https://www.iucnredlist.org/>

Jupe, F., Rivkin, A.C., Michael, T.P., Zander, M., Motley, S.T., Sandoval, J.P., & Ecker, J.R. (2019). The complex architecture and epigenomic impact of plant T-DNA insertions. *PLoS genetics*, 15(1), e1007819.

Kazama, T., Okuno, M., Watari, Y., Yanase, S., Koizuka, C., Tsuruta, Y., & Arimura, S.I. (2019). Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing. *Nature plants*, 5(7), 722-730.

Lange, B.M., Mahmoud, S.S., Wildung, M.R., Turner, G.W., Davis, E.M., Lange, I., & Croteau, R.B. (2011). Improving peppermint essential oil yield and composition by metabolic engineering. *Proceedings of the National Academy of Sciences*, 108(41), 16944-16949.

Lange, I., Poirier, B.C., Herron, B.K., & Lange, B.M. (2015). Comprehensive assessment of transcriptional regulation facilitates metabolic engineering of isoprenoid accumulation in *Arabidopsis*. *Plant Physiology*, 169(3), 1595-1606.

Li, B., Cui, G., Shen, G., Zhan, Z., Huang, L., Chen, J., & Qi, X. (2017). Targeted mutagenesis in the medicinal plant *Salvia miltiorrhiza*. *Scientific reports*, 7(1), 1-9.

Liu, F., Yang, H., Wang, L., & Yu, B. (2016). Biosynthesis of the high-value plant secondary product benzyl isothiocyanate via functional expression of multiple heterologous enzymes in *Escherichia coli*. *ACS synthetic biology*, 5(12), 1557-1565.

Liu T., Yang S.S., Fang R.F., & Zhang G.H. (2015). Establishment of Hairy Root System Mediated by Ri Plasmid and its Advances in Biosynthesis of Plant Secondary Metabolites. *Plant Science Journal*, 33(2), 264-270.

Luo, Y., Li, B.Z., Liu, D., Zhang, L., Chen, Y., Jia, B., & Yuan, Y.J. (2015). Engineered biosynthesis of natural products in heterologous hosts. *Chemical Society Reviews*, 44(15), 5265-5290.

Maher, M.F., Nasti, R.A., Vollbrecht, M., Starker, C.G., Clark, M.D., & Voytas, D.F. (2020). Plant gene editing through de novo induction of meristems. *Nature biotechnology*, 38(1), 84-89.

Muñoz-Bertomeu, J., Arrillaga, I., Ros, R., & Segura, J. (2006). Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant physiology*, 142(3), 890-900.

- Muñoz- Bertomeu, J., Sales, E., Ros, R., Arrillaga, I., & Segura, J. (2007). Up-regulation of an N-terminal truncated 3-hydroxy-3-methylglutaryl CoA reductase enhances production of essential oils and sterols in transgenic *Lavandula latifolia*. *Plant Biotechnology Journal*, 5(6), 746-758.
- Nourozi, E., Hosseini, B., & Hassani, A. (2014). A reliable and efficient protocol for induction of hairy roots in *Agastache foeniculum*. *Biologia*, 69(7), 870-879.
- Nourozi, E., Hosseini, B., & Hassani, A. (2016). Influences of various factors on hairy root induction in *Agastache foeniculum* (Pursh) Kuntze. *Acta Agriculturae Slovenica*, 107(1), 45-54.
- O'Connor, S.E. (2015). Engineering of Secondary Metabolism. *Annual Review of Genetics*, 49, 5.1-5.24.
- Rodrigues, J.L., Araújo, R.G., Prather, K.L., Kluskens, L.D., & Rodrigues, L.R. (2015). Heterologous production of caffeic acid from tyrosine in *Escherichia coli*. *Enzyme and microbial technology*, 71, 36-44.
- Rodriguez Prado, E.A. (2016). Development of a yeast cell factory for production of aromatic secondary metabolites. *The Novo Nordisk Foundation Center for Biosustainability*, 134.
- Sharafi, A., Sohi, H.H., Azadi, P., & Sharafi, A.A. (2014). Hairy root induction and plant regeneration of medicinal plant *Dracocephalum kotschyi*. *Physiology and Molecular Biology of Plants*, 20(2), 257-262.
- Shi, X., Cordero, T., Garrigues, S., Marcos, J.F., Daròs, J.A., & Coca, M. (2019). Efficient production of antifungal proteins in plants using a new transient expression vector derived from tobacco mosaic virus. *Plant biotechnology journal*, 17(6), 1069-1080.
- Trantas, E.A., Koffas, M.A., Xu, P., & Ververidis, F. (2015). When plants produce not enough or at all: metabolic engineering of flavonoids in microbial hosts. *Frontiers in plant science*, 6, 7.
- Tuan, P.A., Kim, Y.S., Kim, Y., Thwe, A.A., Li, X., Park, C.H., & Park, S.U. (2018). Molecular characterization of flavonoid biosynthetic genes and accumulation of baicalin, baicalein, and wogonin in plant and hairy root of *Scutellaria lateriflora*. *Saudi journal of biological sciences*, 25(8), 1639-1647.
- Vuković, R., Bauer, N., & Ćurković-Perica, M. (2013). Genetic elicitation by inducible expression of β -cryptogein stimulates secretion of phenolics from *Coleus blumei* hairy roots. *Plant science*, 199, 18-28.
- Wang, Q., Reddy, V.A., Panicker, D., Mao, H.Z., Kumar, N., Rajan, C., & Sarojam, R. (2016). Metabolic engineering of terpene biosynthesis in plants using a trichome-specific transcription factor Ms YABBY 5 from spearmint (*Mentha spicata*). *Plant biotechnology journal*, 14(7), 1619-1632.
- Wani, S.H., Sah, S.K., Sági, L., & Solymosi, K. (2015). Transplastomic plants for innovations in agriculture. A review. *Agronomy for sustainable development*, 35(4), 1391-1430.
- Weremczuk-Jeżyna, I., Grzegorzczak-Karolak, I., Frydrych, B., Królicka, A., & Wysokińska, H. (2013). Hairy roots of *Dracocephalum moldavica*: rosmarinic acid content and antioxidant potential. *Acta Physiologiae Plantarum*, 35(7), 2095-2103.