Резюме

Оптимізовано середовище для максимального нагромадження ФМН рекомбінантними штамами дріжджів Candida famata, що містять ген РФ-кінази FMN1 під контролем сильного конститутивного промотора TEF1.

Оптимизирован состав среды для максимального накопления ФМН рекомбинантными штаммами дрожжей Candida famata, содержащими ген РФ-киназы FMN1 под контролем сильного конститутивного промотора TEF1.

The medium composition for maximal FMN production by recombinant strains of the yeast *Candida famata* that express the *FMN1* gene encoding riboflavin kinase under control of the strong constitutive *TEF1* promoter was optimized.

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OBTAINING AND ANALYSIS OF TOBACCO, LETTUCE AND RAPE PLANTS TRANSFORMED WITH HUMAN INTERFERON ALFA 2B GENE

Interferons are a large family of multifunctional secreted proteins involved in animal antiviral defence, cell growth regulation and immune system activation [1]. The human interferon alpha2b (hIFN-α2b) is used to treat several diseases including some types of cancer and hepatitis C. Recombinant human interferons have been produced in bacteria, yeast, insect and mammalian cells, and several plant species. Plants as source of pharmaceutical proteins have important advantages over microbial or animal cell systems. They are free from bacterial toxins and human pathogens like viruses and prions, so the recombinant proteins of plant origin are considered to be safer [2]. In some cases they can be used without purification as edible vaccines that lowers production costs considerably [3]. The main obstacle on the way of using transgenic plants for high-scale production of recombinant proteins is the low level of foreign gene expression in case of stable integration into plant nuclear genome (usually about 0.1-0.5 % TSP) [3]. However, the high specific activity of hIFN-α2b may allow using of transgenic plants as food supplements to enhance immune functions of humans and animals.

Here we describe *Agrobacterium*-mediated genetic transformation of model and agricultural plant species with hIFN-α2b gene that resulted in obtaining of plants expressing physiologycally active human interferon.

Materials and methods

Agrobacterium tumefaciens strain GV3101 was used for plant transformation. *Escherichia coli* strain XL1Blue was used for cloning of binary plasmid vectors. Plasmid vectors pICH5290, pCBV16, pICH13301 and pICH17311 were generously donated by Icon Genetics GmbH (Germany). Restriction endonucleases (REs) and T4 DNA ligase were used with supplied buffers (Fermentas, Lithuania). Bacterial cell transformation, plasmid DNA isolation and electrophoretic analysis were carried out as described in [4]. A short nucleotide sequence containing *Bam*HI and *Xba*I recognition sites was added to the pICH13301 and pICH17311 constructs digested with *Pst*I RE. Native and recombinant hIFN-α2b genes were excised with *Nco*I and *Xba*I REs and ligated into pICH5290 and pCBV16 vectors predigested with the same REs. The obtained vectors were designated as pCB73, pCB123, pCB124 and pCB125 (Table1).

Table 1.

Constructed plasmid vectors used for plant trasformation

Plasmid vector	Selective gene	Gene of interest
pCB073	*bar*	Native hIFN-α2b
pCB123	*npt*II	Native hIFN-α2b
pCB124	*npt*II	Recombinant hIFN-α2b with calreticulin signal peptide
pCB125	*bar*	Recombinant hIFN-α2b with calreticulin signal peptide

Nicotiana tabacum cv. Wisconsin was transformed by co-cultivation of leaf explants with *Agrobacterium* carring pCB073 plasmid. Plants were regenerated on MS medium [5] containing 1 mg/l BAP, 0.1 mg/l NAA and 5 mg/l PPT.

Cotyledons and hypocotyls excised from 7-days old seedlings of lettuce (*Lactuca sativa)* cv. Odeskij kucheriavij were inoculated with *Agrobacterium* carring pCB125 plasmid and transferred to B5 medium [6] containing 25 g/l sucrose, 3 mg/l kinetin, 0.5 mg/l naphtylacetic acid, and 5 mg/l PPT. PPT-resistant shoots were rooted on the B5 medium complemented with 0.5 mg/l NAA and 5 mg/l PPT. Obtained plants were transferred to soil and treated with herbicide BASTA.

Rape plants of different varieties (Kletochnyj, Kalinovskij, Westar, Maria, Brutor, Aira, VNIS-100) were transformed by co-cultivation of leaf explants with *Agrobacterium* carring pCB073 or pCB125 plasmid. *Agrobacterium* treated explants were placed onto medium with 2 mg/l 2,4-D, 1mg/l NAA, 0,1 mg/l Kin and 0,1 mg/l BA for callus formation. Plants were regenerated on MS-basal medium supplemented with 2 mg/l BA, 1 mg/l Zea, 1 mg/l NAA, 1 mg/l GA and 1 mg/l ABA containing 10 mg/l PPT [7].

PCR analysis was carried out with primers INTFOR 5'-ctcctgcttgaaggacag-3' and INTREV 5'-ggagtcctccttcatcag-3' amplifying the 264 bp fragment of hIFN-α2b gene. To detect agrobacterial contamination, an additional primer pair (VirD1-1 5'-atgtcgcaaggcagtaagccca-3' and VirD1-2 5'-ggagtctttcagcatggagcaa-3') was simultaneously used for amplification of 432 bp fragment of *vir*D1 gene. The reaction mixture contained 1 mkg of total plant DNA; 0.25 mkM of each primer; 0,5 u of *Taq* DNA polymerase and corresponding buffer (Helicon, Russia); 0.5 mM dNTPs (0.125 мМ each). Amplification was conducted under following conditions: 94°C 5 min → (94°C 30 sec, 60°C 30 sec, 72°C 30 sec) x 30 → 72°C 5 min.

Extracts from plant leaves were prepared in double volume of 100 mM Tris/HCl buffer, pH 8.0, containing 5 mM Na_2EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, and 2.5 % PVP. The total protein content was measured by the method of Bradford. The extracts were tested for their ability to protect cells against cytopathic viral replication in the vesicular stomatitis virus cytopathic effect assay.

Results and discussion

Genetic constructs for *Agrobacterium*-mediated transformation of plant nuclear genome were built up on the basis of binary plasmid vectors pICH5290 and pCBV16. They included *bar* or *npt*II genes, respectively, under control of nopaline synthase promoter and octopine synthase terminator. It allowed selection of transformed plants by their resistance to phosphinothricin (PPT) or kanamycin, respectively. hIFN-α2b gene with native N-terminal cleavable signal peptide and recombinant hIFN-α2b gene fused to *Nicotiana plumbaginifolia* calreticulin signal peptide were derived from pICH13301 and pICH17311 plasmid, respectively. The signal peptides determine translocation of newly synthesized proteins across the membrane of endoplasmic reticulum that leads to their excretion into apoplast and may increase the production level. It was reported previously, that using of *N. plumbaginifolia* calreticulin signal peptide allowed to achieve high level of transient expression of hIFN-α2b gene in *N. benthamiana* [8]. However, we decided to use both native and recombinant hIFN-α2b genes because in different plant species the effectiveness of signal peptides may vary.

The obtained vectors contained the genes of interest under control of CaMV 35S promoter and nopaline synthase terminator (Table 1).

In our experiments we transformed model plant species (tobacco) as well as lettuce and rape which can be used in raw form in human diet or animal feeding. The hIFN-α2b gene was introduced in plant cells by *Agrobacterium* carrying pCB125 or pCB73 vectors. After selection for PPT resistance we have obtained 7 tobacco plants, 9 lettuce plants and 54 rape plants. Presence of the hIFN-α2b gene as well as absence of *Agrobacterium* contamination was confirmed by duplex PCR assay for all obtained tobacco and lettuce plants and for 19 rape plants (12 of variety Kletochnyj, 4 of variety Kalinovskij, 1 of variety Westar, 1 of variety Maria and 1 of variety Brutor).

The extracts from four green-house grown lettuce plants harboring the hIFN-α2b gene were tested for their ability to protect cells against cytopathic viral replication. One of the tested transformants, as well as control non-transformed plant showed no activity in the vesicular stomatitis virus cytopathic effect assay. Three other lines demonstrated varying levels of antiviral activity (Table 2).

Table 2.

Interferon activity in leaf extracts of lettuce plants

Lettuce plant	Interferon activity, IU/ml of leaf extract	Interferon activity, IU/g FW
Non-transformed	0	0
Transformant 1	0	0
Transformant 2	12.5	27
Transformant 3	100	226
Transformant 4	200	448

Primary transformants of tobacco and lettuce were self-fertilized and seeds were obtained.

Physiologically active human interferons have been produced earlier in several plant species by different methods. Stable nuclear transformation was reported for tobacco (interferon beta, [9]), rice plants (interferon alfa, [10]) and cell suspension cultures (interferon gamma, [11]), and potato (interferon α2b and 8, [12]; interferon α2b, [13]). The maximum interferon activity in potato plants reached 560 IU/g FW [12], which is comparable with our data for lettuce (448 IU/g FW, Table 2). In tobacco, high levels (up to 20 % TSP, or 3 mg/g FW) of hIFN-α2b were achieved by chloroplast transformation [14]. However, obtaining of transplastomic plants is a difficult task. Up to date it is feasible for a restricted number of plant species. Many agricultural plants still resist attempts of chloroplast transformation. Another way to increase recombinant protein production in plants is transient expression of foreign gene [15]. By this method active human interferon beta [16] and chicken interferon alpha [17] were produced in lettuce. The drawback of transient expression is the difficulty of scaling up the process. Stable nuclear transformants are suitable for field cultivation in ton range. No additional purification of recombinant protein is necessary if the plant can be freshly consumed. Presently human interferon alfa produced in transgenic duckweed is in phase II of clinical trials (Biolex, USA). In case of hIFN-α2b, the high specific activity of the recombinant protein (10^8 IU/mg) may allow using of transgenic plants even with low level of target protein accumulation as food supplements to enhance immune functions of humans and animals.

Conclusions

We have constructed plasmid vectors for *Agrobacterium*-mediated transformation of plant nuclear genome with human interferon alpha2b gene. With the obtained genetic constructions we performed *Agrobacterium*-mediated transformation of model (tobacco) as well as agricultural plant species (lettuce, rape) and proved the transgen presence in the

selected transformants. The transformed lettuce plants were proved to produce active interferon with the maximal activity about 500 IU/g FW.

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Summary

Plasmid constructs for *Agrobacterium*-mediated transformation of plant nuclear genome with human interferon alpha2b gene were built up and used to obtain transgenic plants of tobacco, lettuce and rape. Presence of the target gene was confirmed by PCR analysis and interferon activity in lettuce plants was shown by the vesicular stomatitis virus cytopathic effect assay.

Сконструированные вектора для *Agrobacterium*-опосредованной трансформации ядерного генома растений геном интерферона альфа 2b человека были использованы для получения трансгенных растений табака, салата и рапса. Присутствие целевого гена было показано с помощью ПЦР анализа. Трансформированные растения салата демонстрировали антивирусную активность интерферона.

Сконструйовані вектори для *Agrobacterium*-опосередкованої трансформації ядерного геному рослин геном інтерферону альфа 2b людини були використані для отримання трансгенних рослин тютюну, салату та ріпаку. Присутність цільового гену було показано за допомогою ПЛР аналізу. Трансформовані рослини салату демонстрували антивірусну активність інтерферону.