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MULTIPLEX PCR ASSAY FOR DETECTION OF HUMAN INTERFERON ALPHA2b GENE IN TRANSGENIC PLANTS

During the last decade interferons are regarded as potent candidates for generation of plant-based edible vaccines because of broad spectrum of antiviral activities and adjuvant properties. Establishment and certification of numerous interferon producing plant systems requests development of fast and efficient multiplex PCR protocol for the transgene detection in GM plants. Here we represent a protocol for simultaneous amplification in one assay of fragments of hIFN alpha 2b gene and two control genes, namely virD1 of Agrobacterium tumefaciens and conservative region of plant actin gene.

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Introduction. Interferons (IFNs) represent an important group of multifunctional proteins coordinating a diverse array of cellular programs in animal organisms. Being a part of the non-specific immune system, they participate in processes of antiviral defence, cell proliferation regulation, signal transduction and immune response modulation [1, 2]. Because of their immunomodulatory effects IFNs are used in treatment of a variety of diseases including several types of cancer, hepatitis C infections, rheumatoid arthritis, osteoporosis etc [1, 3, 4]. Special attention was focused on IFNs as broad spectrum antivirals for prevention or treatment of acute influenza infections [5] and as a potent adjuvant that may be used with both parenterally and mucosally administered vaccines [6].

Plant based recombinant proteins demonstrate correct posttranslational modifications, folding and assembling to multimeric products, e.g. antibodies [7–9]. Some differences in glycosylation patterns of proteins produced in plant and mammalian cells apparently caused no significant effect on their antigen characteristic or specificity [10] and may be overcome by expression in host of human glycosyltransferases [11]. Numerous examples of immune responses in humans or animals caused by oral delivery of plant-based vaccines were reviewed in recent publications [12–14]. Up to now no clear evidence were reported about possible pathogenic action of plant viruses on humans, that makes the recombinant proteins of plant origin safer.

All the mentioned above suggest perspectives of IFN producing transgenic plants as edible vaccines (alone or together with other immunogenic proteins). Apart from bacteria, yeast, insect and mammalian expression systems, recombinant human interferons have been obtained in numerous plant species [15–20], see also [21] for review. It is evidently that further evolution of plant-based vaccine technologies will extend the spectrum of INF producing plants. It requests development of fast and efficient Multiplex PCR (MPCR) protocol for the transgene detection in GM plants. Here we represent a protocol for detection of human interferon alpha2b gene in transgenic plants by simultaneous amplification in one assay of fragments of *hINTα2b* and two control genes, namely *virD1* of *Agrobacterium tumefaciens* and conservative region of plant actin gene. Because PCR techniques as a method for GMO detec-

tion is commonly approved by the regulatory authorities [22], the developed MPCR assay can be recommended for certification of GM plants harboring gene of human interferon alpha2b.

Materials and methods. *Plant material.* Transgenic plants of *Nicotiana tabacum*, *N. benthamiana* and *Brassica napus* carring human interferon α2b (*hINFα2b*) gene under control of 35S CaMV promoter were obtained by *Agrobacterium*-mediated transformation using GV3101 strain with pBIN19-derived binary vectors [23]. Transgenic *Nicotiana* plants transformed with *GFP* gene and rape plants carrying bovine *cyp*11A1 gene coding for cytochrome P450scc were used as a source of nontarget DNA.

DNA isolation. DNA was extracted and purified from the leaf material as described in [24]. The DNA concentration was measured by UV absorption at 260 nm and the purity was evaluated by the $A_{260\ nm}/A_{280\ nm}$ ratio using the BioPhotometer («Eppendorf», Germany).

Primers and PCR conditions. The oligonucleotide primers specific for the sequences given in Table were purchased from Macrochim (Ukraine). The PCR reactions were carried out in 2720 Thermal Cycler («Applied Biosystems», USA). A standard PCR assay (in 10 μl of 1 × PCR buffer consisting of 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01 % Tween 20, 2.5 mM $MgCl_2$) contained 1 μg template plant DNA, 0.5 U *Taq* DNA polymerase («Helicon», Russia), 0.5 mM dNTP, and primers (Table) in concentration 0.25 μM each. The PCR was carried out under following conditions: initial denaturation at 94 °C for 5 min, 30 cycles including denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec, followed by final extension at 72 °C for 5 min.

Restriction endonuclease analysis. For restriction endonuclease analysis PCR products were precipitated with ethanol and digested with *Bgl*II restriction endonuclease («Fermentas», Lithuania) in reaction buffer recommended by the manufacturer.

Agarose gel electrophoresis. The DNA fragments were analysed by electrophoresis in 1 % (w/v) agarose gel in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 150 V for 45 min and visualized with ethidium bromide. The GeneRuler 100 bp DNA Ladder («Fermentas», Lithuania) was used as DNA size marker.

Results and discussion. *Specificity of primers for hINFα2b detection.* Two primer pairs were designed for amplification of human interferon α2b gene (Fig. 1). Native *hINFα2b* encodes the interferon precursor with a signal sequence that defines the secretion of the mature interferon from leukocytes and is cleaved during protein translocation across the endoplasmic reticulum membrane. This sequence supports the same transport in plant cells, but it was reported that substitution of native signal peptide with analogous sequence of plant origin (e.g. derived from calreticulin gene of *N. plumbaginifolia*) leads to significant increase of recombinant interferon levels in plants [19, 27]. Both designed primer pairs are expected to anneal to the part of *hINFα2b* coding for mature interferon that makes them appropriate for detection of native as well as recombinant *hINFα2b* genes fused with different signal sequences. Spec-

List of primer pairs employed for PCR assays

Target sequence	NCBI acc. no.	Primer sequences (5'-3')	Amplicon size (bp)	References
hINFα2b	NT_008413	IntFor ctcctgcttgaaggacag IntRev ggagtcctccttcatcag	265	This study
hINFα2b	NT_008413	IntFor4 ttgatgctcctggcacag IntRev2 ttctgctctgacaacctc	396	This study
Actin gene of *Nicotiana tabacum*	X63603	For tttgctggagatgatgc Rev cttgaatggcgacatac	351	[25]
virD1	M17989	For atgtcgcaaggcagtaagccca Rev ggagtctttcagcatggagcaa	432	[26]

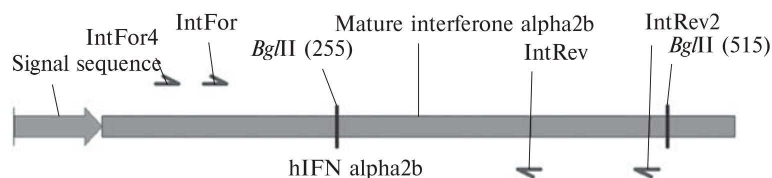

Fig. 1. Scheme of human interferon α2b gene. Arrows indicate the primer positions

ificity of the primer pairs was checked against the GenBank, EMBL and DDBJ databases using the Primer-BLAST algorithm. The designed primer pairs demonstrate homology to interferon α genes of animal origin and no significant similarity with any plant genes that proves their suitability for analysis of transgenic plants.

The simplex PCR assay with the target DNA isolated from transgenic *N. tabacum*, *N. benthamiana* and *B. napus* showed specific *hINFα2b* amplicons of the expected size, i.e. 265 bp with IntFor–IntRev primer pair (results not shown) and 396 bp with IntFor4–IntRev2 oligonucleotides (Fig. 2). The leaves of nontransformed plants of corresponding species served as negative controls. To confirm the amplification specificity, the primer set was used in PCR assay with nontarget tobacco and *N. benthamiana* DNA (containing *GFP* gene) and nontarget rape DNA (with bovine *cyp*11A1 gene), where no amplification was observed. However, further experiments revealed that the primer pair IntFor4–IntRev2 was less efficient in multiplex PCR (results not shown). The multiplex PCR assay was developed using the IntFor–IntRev primer pair.

Additional primer pairs for multiplex PCR analysis. It is advisable to apply for analysis of transgenic plants a multiplex PCR system that includes two control primer pairs. A pair for amplification of an intrinsic plant gene serves as an internal positive control. It allows to reveal false negative results that are due to insufficient DNA quality. Another primer pair should be complement to one of the *Agrobacterium* virulence (*vir*) genes. These genes are necessary for T-DNA transfer into a plant cell but are not transferred themselves. Plant genetic transformation is often carried out using *Agrobacterium*, so the PCR analysis of the primary transformants may show false positive signals derived from the residual agrobacterial contamination of the plant tissues. Amplification of the *vir* gene fragments indicates such false positive results.

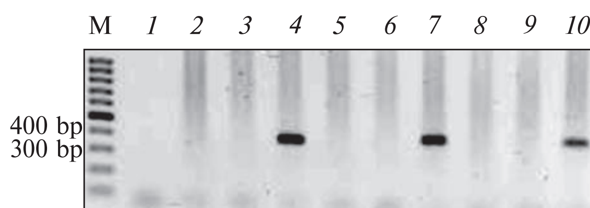

Fig. 2. Detection of *hINTα2b* gene in GM *Nicotiana tabacum*, *N. benthamiana* and *Brassica napus* using the primer pair IntFor4-IntRev2. Lane M – 100 bp DNA ladder. The numbers at the top indicate the template DNA used in each lane: *1* – no template; *2* – non-GM tobacco; *3* – GM tobacco carring *GFP* gene; *4* – GM tobacco carring *hINTα2b* gene; *5* – non-GM *N. benthamiana*; *6* – GM *N. benthamiana* carring *GFP* gene; *7* – GM *N. benthamiana* carring *hINTα2b* gene; *8* – non-GM rape; *9* – GM rape carring *cyp*11A1 gene; *10* – GM rape carring *hINTα2b* gene

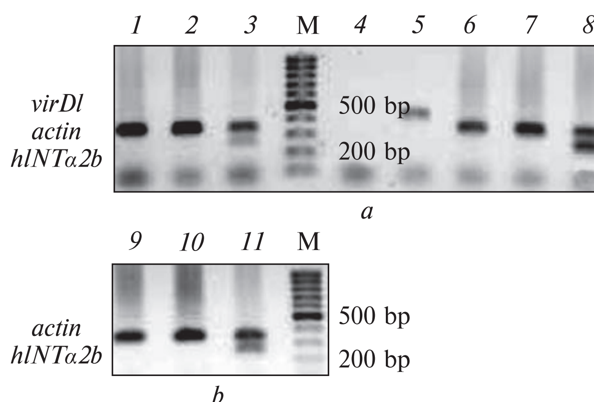

Fig. 3. Triplex PCR detection of *hINTα2b* gene in GM *N. tabacum*, *Brassica napus* (*a*) and *N. benthamiana* (*b*). Lane M, 100 bp DNA ladder. The numbers at the top indicate the template DNA used in each lane: *1* – non-GM tobacco; *2* – GM tobacco carring *GFP* gene; *3* – GM tobacco carring *hINTα2b* gene; *4* – no template; *5* – *A. tumefaciens* GV3101; *6* – non-GM rape; *7* – GM rape carring *cyp*11A1 gene; *8* – GM rape carring *hINTα2b* gene; *9* – non-GM *N. benthamiana*; *10* – GM *N. benthamiana* carring *GFP* gene; *11* – GM *N. benthamiana* carring *hINTα2b* gene

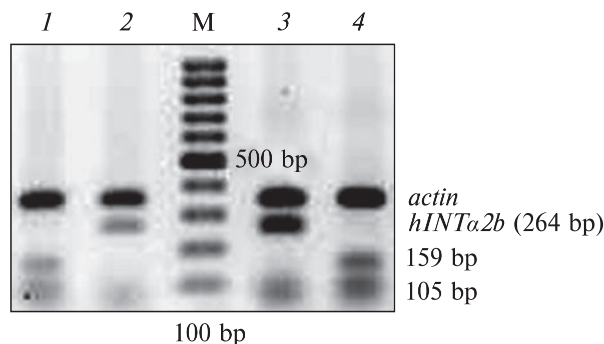

Fig. 4. Restriction fragments of 264 bp *hINTα2b* amplicon. Lane M, 100 bp DNA ladder; *1* – *Bgl*II digested *hINTα2b* PCR product of tobacco DNA; *2* – undigested *hINTα2b* PCR product of tobacco DNA; *3* – undigested *hINTα2b* PCR product of rape DNA; *4* – *Bgl*II digested *hINTα2b* PCR product of rape DNA (actin amplicon of 351 bp remains undigested)

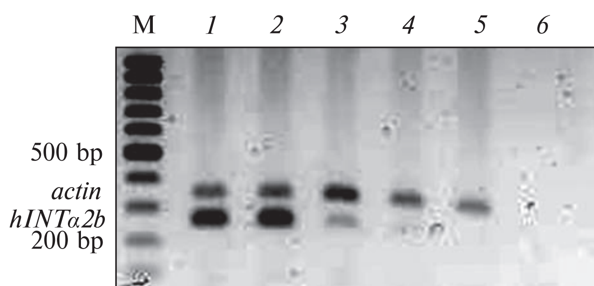

Fig. 5. Detection of *hINTα2b* gene in transgenic *N. benthamiana* DNA spiked with non transgenic DNA. Lane M, 100 bp DNA ladder. The numbers at the top indicate the template DNA used in each lane: *1* – 1 μg of transgenic DNA; *2* – 100 ng of transgenic DNA mixed with 900 ng of non transgenic DNA; *3* – 10 ng of transgenic DNA mixed with 1 μg of non transgenic DNA; *4* – 1 ng of transgenic DNA mixed with 1 μg of non transgenic DNA; *5* – 0.1 ng of transgenic DNA mixed with 1 μg of non transgenic DNA; 6 – no template

We have included in the presented PCR assay the primer pairs for amplification of 351 bp fragment of actin gene and 432 bp fragment of *vir*D1 gene of GV3101 strain of *A. tumefaciens.* These primers were earlier successfully used in MPCR system for detection of recombinant desaturase-lichenase genes in transgenic tobacco, rape and potato plants [25]. Although the primers for amplification of actin gene fragment were designed using the tobacco sequence, they are comple-

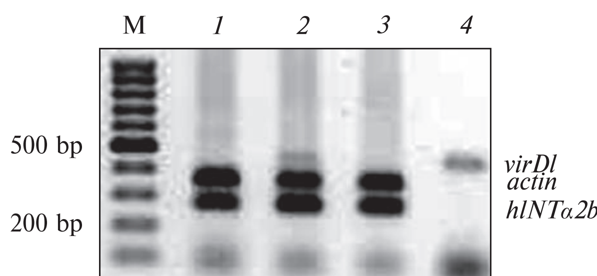

Fig. 6. Detection of A*grobacterium* contamination in transgenic plant DNA. Lane M, 100 bp DNA ladder. The numbers at the top indicate the template DNA used in each lane: *1* – 1 μg of transgenic tobacco DNA; *2* – 1 μg of transgenic tobacco DNA mixed with 5 ng of *A. tumefaciens* GV3101 DNA; *3* – 1 μg of transgenic tobacco DNA mixed with 1 ng of *A. tumefaciens* GV3101 DNA; *4* – 5 ng of *A. tumefaciens* GV3101 DNA

ment to the conserved part of the gene and can be used for analysis of different plant species. We have examined *N. tabacum*, *N. benthamiana* and *B. napus* plants with the developed triplex PCR system. The *hINFα2b* amplicon of 265 bp was observed only with target DNA from plants carrying *hINFα2b* gene, no amplification was detected in non-target transgenic or non-transgenic samples. The 351 bp fragment of actin gene was amplified from all the samples of plant DNA, whereas the 432 bp fragment of *vir*D1 gene was detected only if the DNA of *A. tumefaciens* was used as template (Fig. 3).

Confirmation of the amplicon identity. The amplified *hINFα2b* fragment was subjected to restriction endonuclease hydrolysis in order to prove its identity. The *Bgl*II enzyme was expected to produce fragments of 105 and 159 bp from the 265 bp amplicon (Fig. 1). The experimental data confirmed the identity of the *hINFα2b* fragments obtained from the transgenic tobacco and rape DNA (Fig. 4).

Limit of detection. It is recommended to use 0.5–1 μg of total plant DNA in an assay for transgene presence [24]. The developed MPCR system is able to detect *hINFα2b* when 10 ng of transgenic plant DNA is spiked with 1 μg of non-transgenic DNA that means 1 % of transgenic DNA (Fig. 5). The *virD1* gene is amplified if 5 ng of total *A. tumefaciens* DNA is mixed with 1 μg of transgenic plant DNA (Fig. 6).

Conclusions. The described MPCR assay for detection of *hINFα2b* gene can be applied in course of selection of transformed plants, especially for screening of primary transformants because it allows for detection of *Agrobacterium* contamination. Development of the MPCR method for *hINFα2b* detection is also a necessary prerequisite for legalization of GM plants with antiviral activity carrying this transgene [22].

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МУЛЬТИПЛЕКСНЫЙ ПЦР АНАЛИЗ ПРИСУТСТВИЯ ГЕНА ИНТЕРФЕРОНА АЛЬФА-2b В ТРАНСГЕННЫХ РАСТЕНИЯХ

В последнее десятилетие интерфероны рассматриваются как перспективные кандидаты для получения из растений в виде съедобных вакцин, поскольку обладают широким спектром антивирусной активности и адъювантными свойствами. Создание и сертификация многочисленных растительных систем, продуцирующих рекомбинантный интерферон, делают актуальной разработку быстрого и эффективного протокола мультиплексной ПЦР для определения данного трансгена в генетически модифицированных растениях. В настоящей публикации мы приводим метод детекции гена человеческого интерферона альфа-2b в трансгенных растениях с помощью совместной амплификации в ходе одной реакции фрагментов гена *hINTα2b* и двух контрольных генов, *virD1 Agrobacterium tumefaciens* и консервативного участка гена актина растений.

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МУЛЬТИПЛЕКСНИЙ ПЛР АНАЛІЗ ПРИСУТНОСТІ ГЕНА ИНТЕРФЕРОНУ АЛЬФА-2b В ТРАНСГЕННИХ РОСЛИНАХ

В останнє десятиліття інтерферони розглядаються як перспективні кандидати для отримання з рослин у вигляді їстівних вакцин, оскільки вони мають широкий спектр антивірусної активності й ад'ювантні властивості. Створення і сертифікація численних рослинних систем, які накопичують рекомбінантний інтерферон, роблять актуальною розробку швидкого й ефективного протоколу мультиплексної ПЛР для визначення даного трансгена в генетично модифікованих рослинах. В цій публікації ми наводимо метод детекції гена людського інтерферону альфа-2b у трансгенних рослинах за допомогою сумісної ампліфікації в ході однієї реакції фрагментів гена *hINTα2b* і двох контрольних генів, *virD1 Agrobacterium tumefaciens* і консервативної ділянки гена актину рослин.

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