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Short Exogenous Peptides Regulate Expression of *CLE*, *KNOX1*, and *GRF* Family Genes in *Nicotiana tabacum*

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Abstract—Exogenous short biologically active peptides epitalon (Ala-Glu-Asp-Gly), bronchogen (Ala-Glu-Asp-Leu), and vilon (Lys-Glu) at concentrations 10^{-7}-10^{-9} M significantly influence growth, development, and differentiation of tobacco (*Nicotiana tabacum*) callus cultures. Epitalon and bronchogen, in particular, both increase growth of calluses and stimulate formation and growth of leaves in plant regenerants. Because the regulatory activity of the short peptides appears at low peptide concentrations, their action to some extent is like that of the activity of phytohormones, and it seems to have signaling character and epigenetic nature. The investigated peptides modulate in tobacco cells the expression of genes including genes responsible for tissue formation and cell differentiation. These peptides differently modulate expression of *CLE* family genes coding for known endogenous regulatory peptides, the *KNOX1* genes (transcription factor genes) and *GRF* (growth regulatory factor) genes coding for respective DNA-binding proteins such as topoisomerases, nucleases, and others. Thus, at the level of transcription, plants have a system of short peptide regulation of formation of long-known peptide regulators of growth and development. The peptides studied here may be related to a new generation of plant growth regulators. They can be used in the experimental botany, plant molecular biology, biotechnology, and practical agronomy.

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The short exogenous biologically active peptide epitalon increases lifespan of experimental animals [1]. Bronchogen regulates cell differentiation, proliferation, and apoptosis in cultures of cells of human bronchial epithelium, similar to actions of vilon in thymus cells and human and animal lymphocytes [2]. These peptides induce tissue-specific expression of genes including genes responsible for DNA replication and repair [2, 3]. The action of epitalon, bronchogen, and vilon in plant cells is unknown.

It was suggested that the physiological action of these peptides in animals is based on their gene-specific interaction with DNA due to specific (complementary) peptide–DNA binding [2-4].

We proposed that some general principles of regulatory action of short peptides on gene expression and cell differentiation in eukaryotes (animals and plants) may be quite common. In our opinion, the short peptides mentioned active in animal cells could or even should possess regulatory (signaling) action in plant cells also. It was necessary to learn first whether these peptides influence somehow plant growth and development. Therefore, we investigated how and to what extent these peptides influence some morphological features of tobacco plant calluses and expression of some key genes controlling tobacco cell differentiation.

The goal of the present work was to investigate the influence of short exogenous peptides on growth and development of tobacco callus cultures and expression of some key regulatory genes responsible for cell differentiation, growth, and development in plants. Tetrapeptides epitalon (Ala-Glu-Asp-Gly) and bronchogen (Ala-Glu-Asp-Leu) and the dipeptide vilon (Lys-Glu) synthesized in the St. Petersburg Institute of Bioregulation and Gerontology were used in this work.

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MATERIALS AND METHODS

Seeds of tobacco plants (*Nicotiana tabacum* L., Samsun variety) were sterilized by 1.5% sodium hypochlorite solution containing 0.01% Triton X-100 for 15 min. Then the seeds were washed three times with sterile distilled water and placed into flasks with Murashige–Skoog agar medium for germination. Cotyledons formed after seed germination were cut off with a scalpel and placed into Petri dishes on Murashige–Skoog agar medium containing 6-benzylaminopurine (1 mg/liter), indolylbutyric acid (0.2 mg/liter), and one of the peptides (vilon, bronchogen, epitalon) added to concentrations from 10^{-6} to 10^{-9} M or without peptides. Explants growing in Petri dishes were incubated in darkness in a thermostat at 25°C for 14 days, and then they were grown in a light room (16 h light period), the room temperature being 22°C (day time) and 16-18°C (night). Experiments were repeated four times.

RNA from tobacco calluses was isolated using reagent kit RNA Extran (Syntol, Russia) by the procedure described by the manufacturer. Concentrations of nucleic acid preparations were measured spectrophotometrically. **cDNAs** were obtained by a standard method using a reagent kit for reverse transcription (Syntol).

Data on primary structures of genes *CLE*, *KNOX1*, and *GRF* of *N. tabacum* and *N. sylvestris* were obtained from the NCBI database. The respective primers for gene transcripts were selected by online service NCBI Primer-BLAST and synthesized by Syntol (Tables 1-3).

Real time PCR was performed in a CFX 96 Real-Time System thermocycler (BioRad, USA). The samples were prepared using the reagent kit for RT-PCR (Syntol) in the presence of EVA Green (Syntol). RT-PCR was performed under the same conditions for all samples: 95°C – 5 min (DNA polymerase activation), then 45 cycles (94°C – 30 sec, 58°C – 30 sec, 72°C – 30 sec). After the last step, the reaction mixtures were incubated for 2 min at 72°C. All PCR runs were repeated thrice. The relative levels of gene transcription were determined using respective calibration curves obtained with PCR products formed with primers to the *GAPDH* gene.

Statistical data treatment. Mean values were calculated using the formula $M_x = \Sigma Xi/n$. Standard deviation values were calculated as:

$$\sigma_x = \sqrt{Dx} = \sqrt{\Sigma(Xi - M_x)^2}/(n - 1),$$

where Dx is dispersion. The dispersion and standard deviation values were calculated using the Excel program.

RESULTS AND DISCUSSION

Growing tobacco calluses (*N. tabacum* L., Samsun variety) on standard medium in the presence of 10^{-7}-10^{-9} M peptides resulted in increase in growth rate and callus mass formation compared with control (Fig. 1). Growth of calluses at the higher peptide concentration (10^{-6} M) was suppressed. At very low peptide concentra-

Table 1. *CLE* genes and their primers

Gene	Primer	Encoded protein	Protein function
CLE-1	tcg tgg act tga gag cat gag ggt cct cca ggt gct act ct	PREDICTED: CLAVATA3/ESR (CLE)-related protein **5**-like	inhibits cytokinin signaling
CLE-2	aag agt aac cag cct gcc ac gcc aag aac aaa ggg tgc tg	PREDICTED: CLAVATA3/ESR (CLE)-related protein **40**	stimulates ABA biosynthesis, inhibits cytokinin signaling, selectively regulates auxin signaling
CLE-3	gct atc ggg gcc ttg aaa gta tcc tcc agg tgc cac tct at	PREDICTED: CLAVATA3/ESR (CLE)-related protein **6**-like	stimulates proliferation of procambial cells, compensates deficiency of gibberellic acid
CLE-4	tct cca cga gat agg ggc aa gag acc aac tgc aat gcc ac	PREDICTED: CLAVATA3/ESR (CLE)-related protein **44**	
CLE-5	tta cca cca cca caa aca cga tgg tcc aga cgg tac gag ac	PREDICTED: CLAVATA3/ESR (CLE)-related protein **12**-like	inhibits growth of lateral roots
CLE-6	act tga caa agc aaa att ggt tgc tcc atc gga tct gga cca ct	PREDICTED: CLAVATA3/ESR (CLE)-related protein **18**-like	
CLE-7	cac aag aca gca ggg atc aa gct cga gcg att ggg atc aa	PREDICTED: CLAVATA3/ESR (CLE)-related protein **45**	regulates growth of pollen tubes and seed development
CLE-8	aag gaa aac tca gcg agc ca cca ctg act cta cct ggc ct	PREDICTED: CLAVATA3/ESR (CLE)-related protein **25**-like	

Table 2. *KNAT* and *LET* genes and their primers

Gene	Primer	Encoded protein
KNAT1	caa ctc agc gac ctc atg ga/tgt tcc cat ggg cct tca tc	homeobox protein knotted-1 like 1
KNAT2	cgc cat att ttg gat cgc cg/ccg aac aca ccg acg aca ta	homeobox protein knotted-1 like 2
KNAT3	cgt gtg agg cag gag cta aa/agt atc gcc cgg gag ttt tc	homeobox protein knotted-1 like 3
KNAT6	gct gta gca gac gcg atg at/tct ggt ggt gct cct acc tt	homeobox protein knotted-1 like 6
LET6	act tcc tcc tct gaa tct gct/c tgc gca gca att gac ctt tc	homeobox protein knotted-1 like LET6
LET12	agt gca aga gac agg gtt gc/ttt ttc acc tct ttc gtt tgc tt	homeobox protein knotted-1 like LET12

Table 3. *GRF* genes and their primers

Gene	Primer	Encoded protein
GRF-1	ccc gga ttc cca act aca ca/agc gcg tgt act tca cta ctt	DNA-(apurinic or apyrimidinic site) lyase 2-like
GRF-2	cat cca gca gtg cac aga ga/ctt cct gag acc gag cag tg	DNA topoisomerase 3-alpha-like
GRF-3	tac gaa ctg tga ggc atc cg/ttc acc act caa tgt gcc gt	3'-5' exoribonuclease 1-like
GRF-4	gac gaa gag gaa ggc ttg ga/gcc gta ctc cca tca gct tt	endonuclease 8-like 3

tion in the medium (10^{-9} M), the peptide growth stimulating effect was observed also but it was less expressed compared with callus growth at 10^{-8} M peptide concentration. Therefore, this peptide concentration (10^{-8} M) in the medium was mostly used in our experiments on callus grown for investigation of the influence of peptide on gene expression in tobacco calluses. In the presence of peptides, the formation and growth of leaves were increased, and sometimes root formation was increased also. Rate of leaf formation and callus growth in the presence of bronchogen were higher than that in the presence of epitalon and vilon. The effectiveness of growth stimulating action of the peptides can be represented in the order – bronchogen > epitalon > vilon.

Thus, the investigated peptides exert a physiological action on plants. As they are efficient at very low concentrations, their action may to some extent be like the action of phytohormones, and it seems to have signaling character. This is in an agreement with data on the presence in plants of very many relatively short (2-100 amino acid residues) peptide hormones [5-7].

Like phytohormones, secreted peptides play a significant role in regulation of intercellular interactions, physiological activities, and cell responses to various signals of the medium [5-7]. Peptides of the CLE family (CLAVATA3/Endosperm surrounding region-related) are the most investigated secretory peptides in plants [5-7]. The *CLE* genes code for relatively small secreted peptides with conservative C-end motifs. In *Arabidopsis thaliana* plants, 32 peptides of the CLE family were found, but only a few *CLE* genes have been functionally characterized [7]. Endogenous peptides work in cooperation with phytohormones [8, 9], and they take part in regulation of cell physiological activities and biological processes by signals from the external medium. CLE peptides participate in regulation of seed development, formation of the vascular system, lateral roots, and growth of pollen tubes. They control homeostasis of stem cells in the seedling apical and root meristems [5-7]. In tobacco plants, the functional role of CLE peptides and their possible interaction with phytohormones and other plant growth regulators are practically unknown.

Data on some known peptides of the CLE family of *N. tabacum* and on functional activities of similar peptides in *A. thaliana* [9] are presented in Table 1. Comparative analysis of data on the functional activity of CLE peptides in various plants suggest that in tobacco plants (*N. tabacum*) these peptides may play similar roles.

Data on expression of *CLE* genes in tobacco calluses in the presence of short exogenous peptides are shown in Fig. 2. Epitalon and bronchogen are acidic tetrapeptides (Ala-Glu-Asp-Gly and Ala-Glu-Asp-Leu, respectively) that are similar in primary structure, but vilon (Lys-Glu) is a neutral dipeptide that is different in primary structure compared with the two peptides mentioned above. Therefore, it could be expected that the action of vilon on

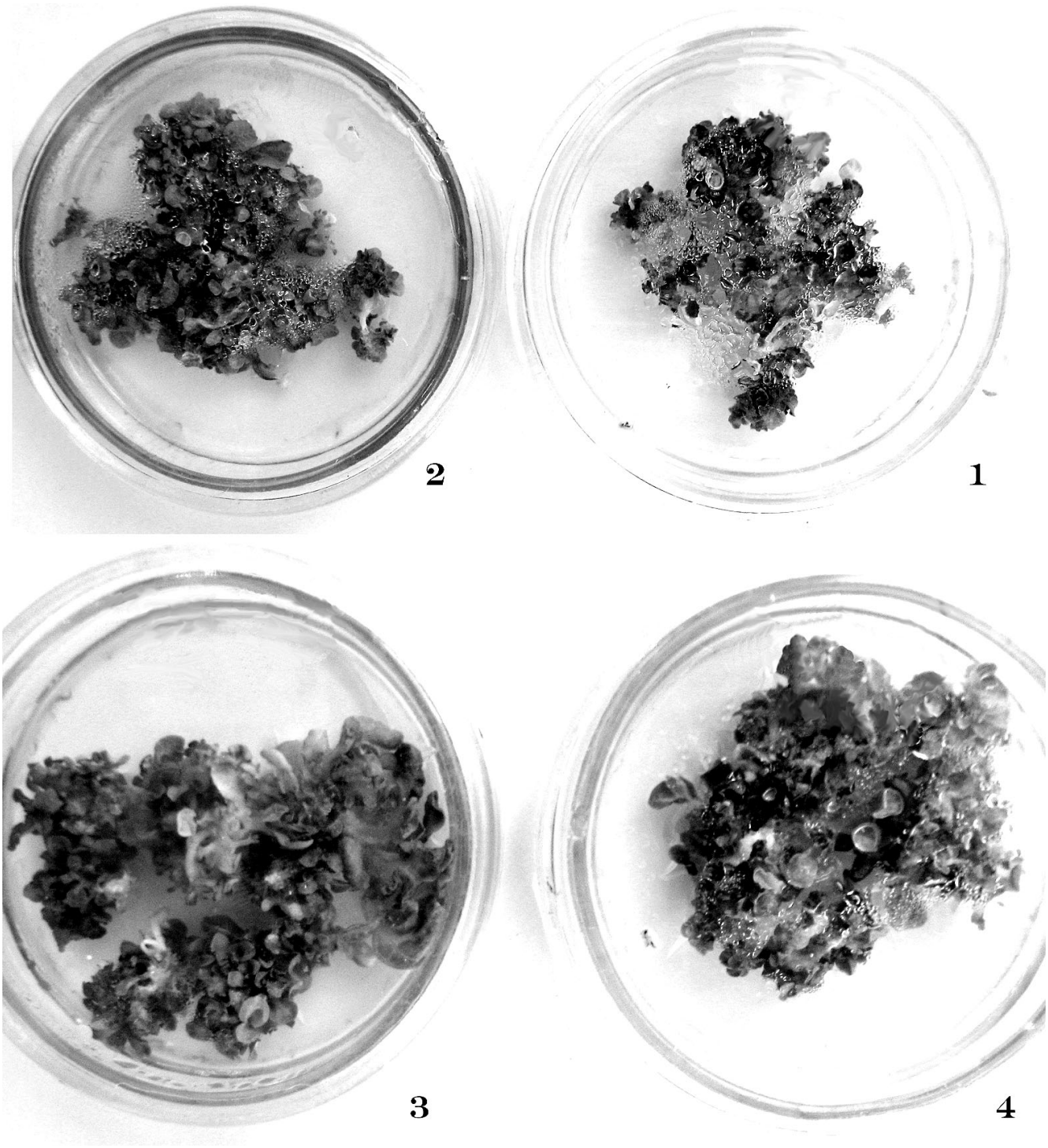

Fig. 1. Influence of peptides at concentration 10^{-8} M on growth of tobacco callus cultures: *1*) control; *2*) epitalon; *3*) bronchogen; *4*) vilon.

gene expression in tobacco calluses will be different compared with actions of bronchogen and epitalon. Vilon significantly suppressed expression of *CLE* genes except for *CLE6*, whose expression under the influence of vilon was increased almost twofold. The three peptides differently affect expression of *CLE* genes in tobacco plants. Thus, the action of the peptides in plants is gene selective as already observed in animals [1]. Both tetrapeptides increase expression of the *CLE2*, *CLE5*, and *CLE6* genes. Expression of the *CLE1* gene is markedly inhibited by epitalon, and it was almost unchanged in the presence of bronchogen and vilon. Epitalon significantly inhibits but

vilon practically completely blocks expression of the *CLE4* gene. In contrast, bronchogen increased expression of this gene almost twofold. Both tetrapeptides had practically no influence on expression of the *CLE3* gene, but vilon decreased it. Thus, short exogenous peptides influence expression of genes coding for endogenous regulatory peptides (CLE), and this action depends on the nature (primary structure) of the exogenous peptides.

We observed earlier that short exogenous peptides can penetrate into animal cells and nuclei, and, therefore, they might interact with chromatin and its structural elements [10]. It was suggested that peptides might bind to gene promoters [4, 10]. It seems that this may be true for the interaction of peptides with *CLE* genes in plants also that may thus result in changes in the expression levels of these genes.

Proteins of the class KNOTTED1-like homeodomain (KNOX) are crucial homeostasis regulators of stem cells in plants. *KNOX* genes code for transcription factors that stop cell differentiation in the seedling apical zone, and these proteins are found in all monocots and dicots [11]. Ectopic expression of *KNOX* genes in different plants influences the phytohormone level and induces dramatic changes in leaf and flower morphologies [11].

The *KNOX* family genes *KNAT1*, *KNAT2*, *KNAT6*, and *STM* (in *Nicotiana* *LET6* and *LET12* are homologues of *STM* genes) (Table 2) participate in stem cell differentiation. Expression of *STM* gene results in early formation of leaves in callus cultures. In *Arabidopsis*, formation of leaf cells is regulated by all *KNOX* genes [12].

Judging from the action of the studied peptides on gene expression, the *KNOX* genes can be divided in three groups (Fig. 3). First group includes the *KNAT1* and *KNAT2* genes, whose expression does not change in the presence of the short peptides. The second group (*KNAT3* and *KNAT6*) contains genes whose transcription is modulated by all the studied peptides. Epitalon increases transcription of *KNAT3* gene almost twofold and bronchogen fourfold. The expression level of the *KNAT6* gene under action of epitalon increases by more than four times, but bronchogen and vilon increase transcription of this gene by six or more times. In the third gene group (*LET6* and *LET12*), the transcription of the *LET6* gene was not changed by epitalon and was slightly stimulated by bronchogen and vilon. Expression of the *LET12* gene was markedly (by 1.6-fold) stimulated by bronchogen and very slightly stimulated by epitalon and vilon.

It is known that in *Arabidopsis* the *STM* gene is essential for leaf formation. Formation of leaves was increased in tobacco callus in the presence of bronchogen (Fig. 1). This correlates with a proposal that *LET6* and *LET12* genes are responsible for leaf differentiation. Vilon also stimulates leaf formation in tobacco callus culture. In fact, vilon increases transcription of the *KNAT3* and *KNAT6* genes (Fig. 3). Thus, in tobacco plants *KNAT3* and *KNAT6*, as well as the *LET6* and *LET12* genes, may be responsible for leaf formation.

Regulation of transcription of *KNOX* genes is controlled by many factors and agents that modulate structural and functional organization of chromatin [13]. It is known that short peptides can bind specifically to chromatin and influence its enzymatic modifications and interactions with transcription factors, enzymes of DNA synthesis, and other proteins and RNA. We established earlier that short peptides can specifically interact with histones H1 and core histones [14]. Bronchogen and epitalon seem to bind to positively charged motif **kaakakk**, but vilon, containing a lysine residue, is most likely to bind to negatively charged motif **evaa**. It was suggested that specific binding of short biologically active peptides to histones may modulate the action of various enzymes on chromatin histones and significantly influence many

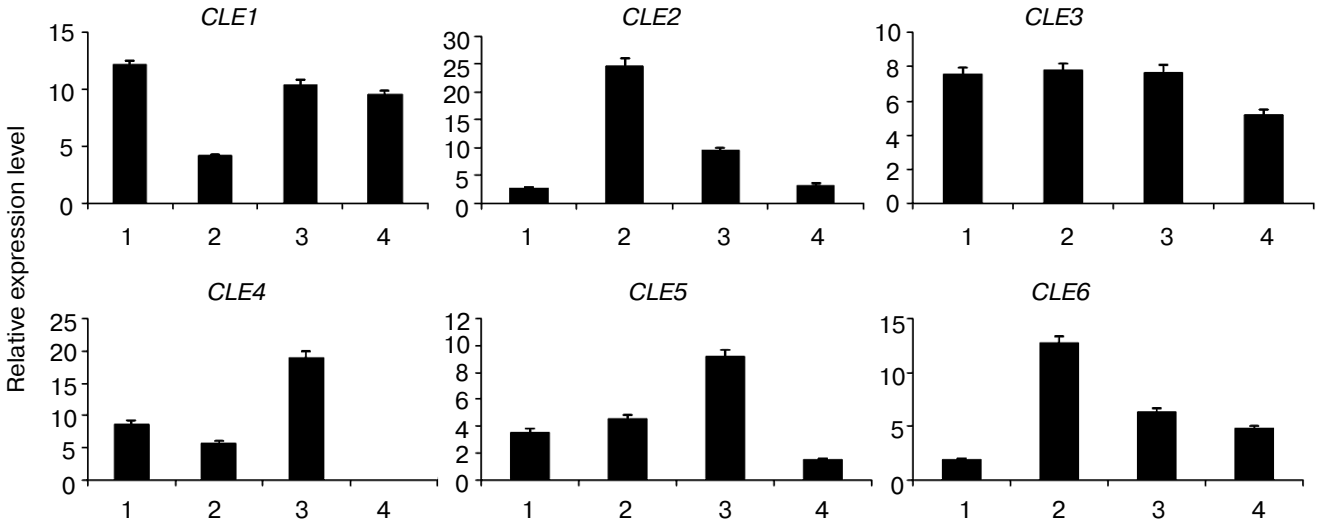

Fig. 2. Effect of peptides on expression of *CLE* family genes: *1*) control; *2*) epitalon; *3*) bronchogen; *4*) vilon.

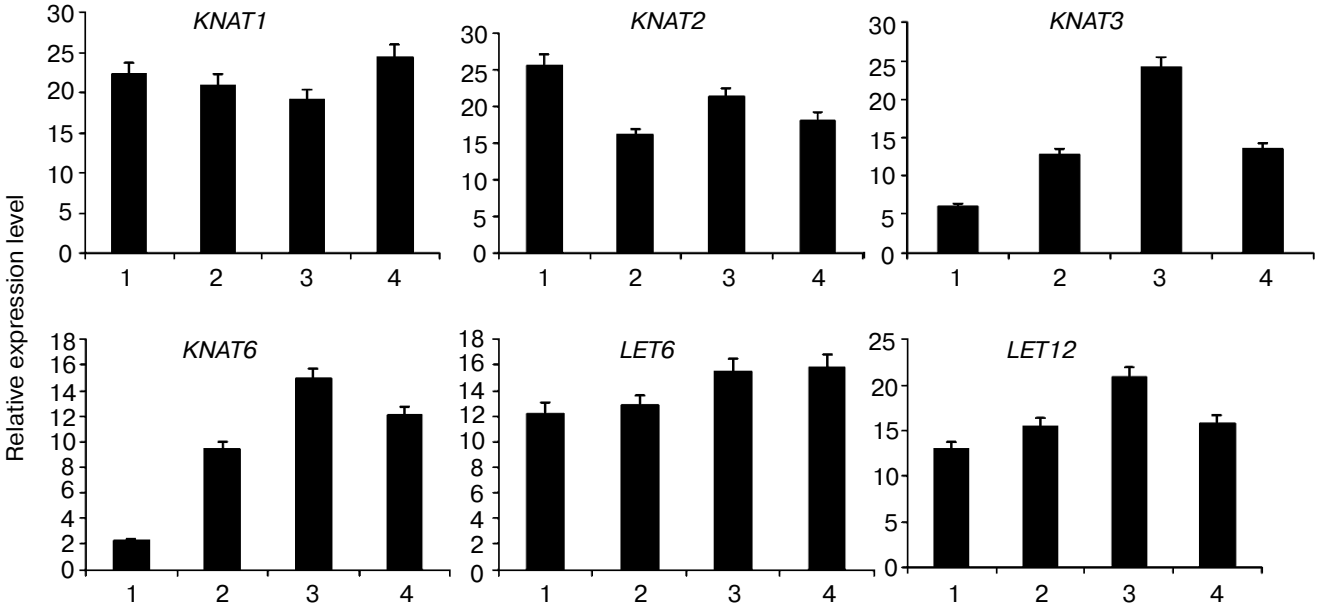

Fig. 3. Effect of the peptides on expression of *KNOX1* family genes: *1*) control; *2*) epitalon; *3*) bronchogen; *4*) vilon.

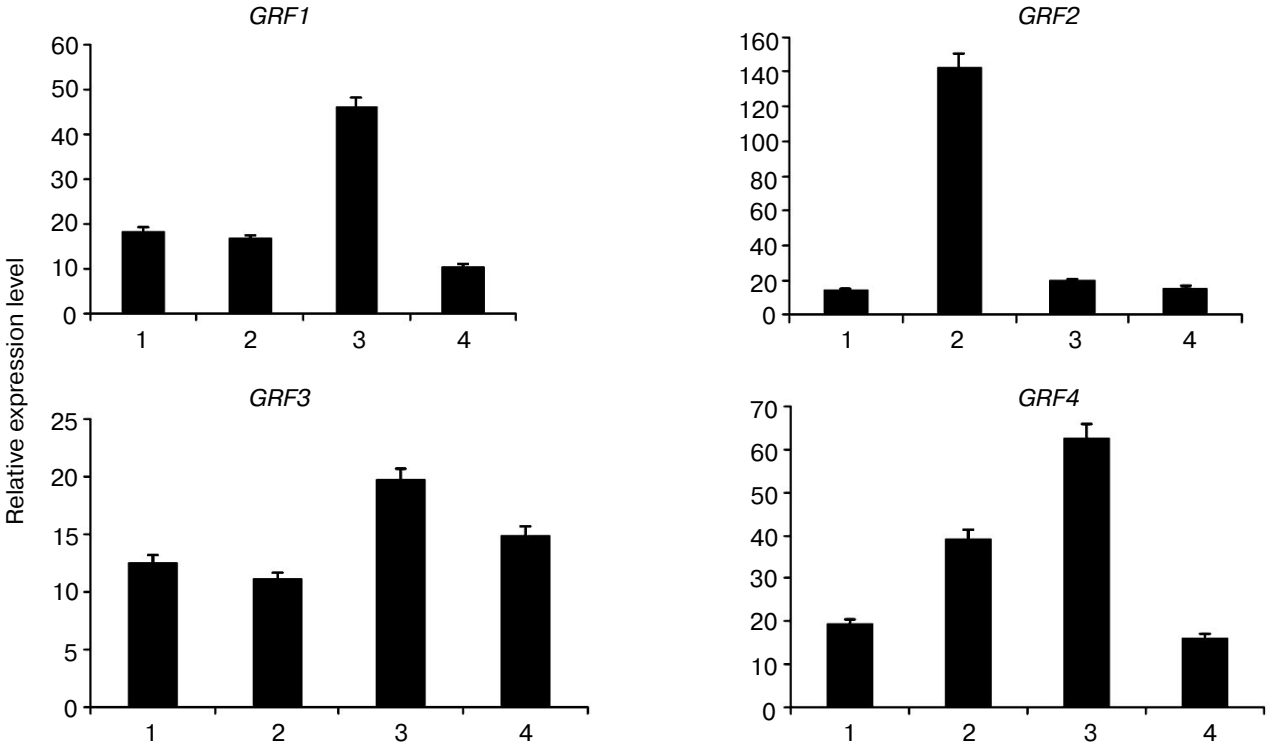

Fig. 4. Effect of the peptides on expression of genes coding for plant growth regulatory factors: *1*) control; *2*) epitalon; *3*) bronchogen; *4*) vilon.

known enzymatic modifications of histone "tails". Thus, protein–protein interactions of short peptides with histones in chromatin may serve as a control mechanism of epigenetic regulation of gene activity and cell differentiation.

Plant growth regulatory factors (GRF) are mainly specific transcription factors [15]. While not so many GRF genes are present in tobacco plants (not more than 20), the respective encoded proteins for only four genes have been established. This was a main reason for our

choice of respective GFR genes for investigation of peptide action on their expression. All proteins encoded by these genes bind with specific regions of DNA. Among such proteins are apurinic/apyrimidinic ligase, DNA topoisomerase 3α, 3′,5′-exonuclease, and endonuclease 8 (Table 3). As in the NCBI there are no data on *GRF* genes of *N. tabacum*, we used the respective gene sequences of *N. sylvestris*. Primers for *GRF* gene transcripts of *N. sylvestris* were efficiently used for amplification of cDNA fragments of *N. tabacum* (Table 3 and Fig. 4).

In tobacco calluses, bronchogen increased transcription of the *GRF1* by more than twofold, but epitalon and vilon slightly inhibited it. Expression of the *GRF2* gene under the influence of epitalon increased by almost 10-fold, while bronchogen and vilon did not influence it. Expression of the *GRF3* gene was stimulated by bronchogen by 1.6-fold and was practically unchanged by epitalon and vilon. Finally, *GRF4* gene expression was increased by epitalon and bronchogen by 2- and 3-fold, respectively, with no influence of vilon. Thus, short exogenous peptides influence the expression of genes coding for plant growth regulatory proteins, and this action depends on the primary structure of the peptide.

It is known that peptide GRF7 of *A. thaliana* binds to DNA core sequence TGTCAGG [16]. In the promoter of the *Oskn2* gene from the rice gene family *KNOX1* (KNOTTED-LIKE HOMEOBOX), homologous motifs were found. It was established that subfragments binding peptides OsGRF3 and OsGRF10 are enriched with CAG sequence that is inside the core sequence TGT**CAG**G. It seems that rice GRF binds to CAG or CTG in promoters [13]. During interaction with deoxyribooligonucleotides, epitalon and bronchogen bind predominantly to the CNG motif [10]. It seems that in tobacco plant cells the short exogenous peptides may also bind to gene promoters at CAG and CTG sites and change expression of these genes. Interestingly, these sequences are target sites for cytosine DNA methylation in plants [17]. Perhaps peptide binding to these DNA sequences influences the level of CNG site methylation in DNA.

Thus, short biologically active peptides influence growth, development, and differentiation of tobacco callus cultures. They act in very low concentrations and modulate expression of various genes including genes responsible for cell differentiation. Calculated constants of peptide binding with oligonucleotides and DNA *in vitro* are about 10^{-8}-10^{-9} M [10], and these values are similar to constants of binding of individual components in hormone–receptor complexes. In combination with gene-specific action of the studied peptides, this may show that the action of the peptides is to some extent similar to the action of phytohormones. Unfortunately, we do not know whether receptors for these peptides exist in plant cells. Nevertheless, it is well known that these peptides penetrate into animal cells and are detected in the nucleus and nucleolus [10]. As mentioned, they might interact with different cell structures and components including DNA and RNA. Let us propose that such short peptides acting gene specifically in animal and plant cells could be evolutionally early ones and common for eukaryotes. It is probable that at the early stages of evolution they could be functionally active without specific receptors. If such receptors appeared later in evolution, they should be quite similar in plants and animals. A special search for such receptors for the studied peptides is of interest.

It is very important to emphasize that we have observed for the first time a control function of short exogenous peptides that regulates expression of genes encoding plant peptide hormones. In other words, short exogenous peptides control expression of endogenous regulatory peptides. Therefore, special short peptide regulators are found for natural peptide regulators. This suggests that such or similar short peptides consisting of 2-4 a.a. formed in plant cells act similarly to phytohormones. Their action has mainly signaling character and is probably of epigenetic nature. Anyway, the investigated short peptides may be considered as perspective plant growth regulators of a new generation. They can be used in plant molecular biology, experimental botany, and practical plant cultivation. Unfortunately, the molecular mechanisms of short peptide action on gene expression are still unknown. It can be suggested that they could act epigenetically, for example, by binding with respective gene promoters and possibly inhibit promoter methylation.

Thus, the studied short peptides are physiologically active in plants and animals. They mainly control growth, development, and cell differentiation by regulation of gene expression. This suggests the existence of common mechanisms of peptide regulation in eukaryotes and evolutionally early origin of such common regulators and respective regulatory systems.

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