

Influence of Glycylglycine, Glycine, and Glycylaspartic Acid on Growth, Development, and Gene Expression in a Tobacco (*Nicotiana tabacum*) Callus Culture

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Abstract—The dipeptides glycylglycine (GlyGly) and glycylaspartic acid (GlyAsp) and amino acid glycine (Gly) in a concentration of 10^{-7} M in a medium essentially stimulate the growth and development of tobacco (*Nicotiana tabacum*) calli. GlyGly, GlyAsp, and Gly influence the cell differentiation and tissue formation processes. They stimulate formation and growth of leaves and roots. After incubation of tobacco seedling roots in the presence of 10^{-5} M fluorescent FITC-labeled peptides or glycine, marked fluorescence was observed in cells of the root cap and epidermis. The fluorescence was detected in the cell walls, cytoplasm, and nuclei. Thus, the peptides used can penetrate into the plant cell and be located in the nucleus and other cell compartments. Therefore, they may potentially interact with different structures and components of the cytoplasm and nucleus including various proteins, RNAs, and DNA. The penetration and accumulation of peptides in cells are tissue specific. In the tobacco callus, peptides modulate expression of the *KNOX* and *GRF* family genes that are responsible for cell differentiation and code for transcription factors. Thus, the dipeptides GlyGly and GlyAsp and amino acid Gly have marked physiological activity and can be related to efficient plant growth regulators.

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INTRODUCTION

Short exogenous biologically active peptides increase the lifespan of animals and significantly improve the physiological status of older people (Khavinson and Malinin, 2005). They selectively stimulate gene expression and protein synthesis, including those involved in DNA replication and repair and are responsible for cellular differentiation (Khavinson and Malinin, 2005). The action of such peptides is gene-specific, it has a signal regulatory nature and, apparently, is mainly epigenetic (Vanyushin et al., 2017). The regulating effect of short exogenous peptides was found in both animals and plants (Khavinson and Malinin, 2005; Vanyushin et al., 2017).

In plants, as in animals, short peptides induce the expression of genes encoding transcription factors responsible for cell differentiation, growth, and development (Vanyushin et al., 2017). It was assumed that the peptide regulation of life in eukaryotes is the same and that it originated during the early stages of evolution (Vanyushin et al., 2017). Therefore, investigation of the effect of the simplest short peptides, which could have appeared as a result of abiogenic processes

during the early stages of the formation of life, on different organisms and biological systems, is of considerable interest. Such peptides include glycylglycine (GlyGly) and glycylaspartic acid (GlyAsp), consisting of only two amino acid residues. Unlike other short peptides (Vanyushin et al., 2017), the effect of these simple peptides on the growth and development of plants is unknown.

The main goal of this work was to investigate the influence of low concentrations of the dipeptides GlyGly and GlyAsp and amino acid glycine (Gly) on the growth, development, and expression of genes in a tobacco (*Nicotiana tabacum*) callus culture.

MATERIALS AND METHODS

Seeds of tobacco plants (*Nicotiana tabacum* L., Samsun variety) were sterilized with a 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 a Murashige–Skoog (MS) agar medium without hormones for germination. Cotyledons formed after seed

Table 1. Primers for *KNOX* genes of *Nicotiana tabacum*

| Gene | 5'–3'-sequence | Encoded protein |
|--------------|--|--|
| <i>KNAT1</i> | caa ctc agc gac ctc atg ga tgt tcc cat ggg cct tca tc | Homeobox protein knotted-1 like 1 |
| <i>KNAT2</i> | cgc cat att ttg gat cgc cg ccg aac aca ccg acg aca ta | Homeobox protein knotted-1 like 2 |
| <i>KNAT3</i> | cgt gtg agg cag gag cta aa agt atc gcc cgg gag ttt tc | Homeobox protein knotted-1 like 3 |
| <i>KNAT6</i> | gct gta gca gac gcg atg at tct ggt ggt gct cct acc tt | Homeobox protein knotted-1 like 6 |
| <i>LET6</i> | act tcc tcc tct gaa tct gct c tgc gca gca att gac ctt tc | Homeobox protein knotted-1 like LET6 |
| <i>LET12</i> | agt gca aga gac agg gtt gc ttt ttc acc tct ttc gtt tgc tt | Homeobox protein knotted-1 like LET12 |

Table 2. Primers for *GRF* genes of *Nicotiana tabacum*

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

germination were cut off with a scalpel and placed into Petri dishes on an MS agar medium containing 10^{-7} M GlyGly, GlyAsp, or Gly (Serva, United States) or on the same medium without the peptides and amino acid. In the experiment 4–5 Petri dishes (replicates) were used. The medium also contained phytohormones: 1 mg of 6-benzylaminopurine (BAP) and 0.2 mg of indole-acetic acid (IAA). Petri dishes with explants were kept in a thermostat in the dark at 25°C for 14 days and then placed in a climatic chamber. Explants were cultivated in the daytime at 20–22°C, and at night at 16–18°C (16 h light period). The experiments on the cultivation of calli were performed in four replicates. Explants were grown for 21 days, and the frequency of callusogenesis and the morphology of the calli formed were recorded: color, texture, callus volume, number of formed regenerants, and leaves. At the end of the experiment (after 28 days), normally formed plants (regenerants) with shoots and a root system and forms with developmental abnormalities (gemmagenesis) were detected. The efficiency of regeneration was calculated as the percentage of calli forming normal regenerating plants from the total number of callus lines. The fresh weight was used as one of the characteristics of calli.

RNA from individual regenerants of a tobacco callus was isolated by the standard method using the reagent kit RNA-Extran (Synthol, Russia). The concentration of isolated RNA preparations was determined spectrophotometrically.

cDNA was obtained according to a standard procedure using a reagent kit (Synthol) for reverse transcription.

Data on the primary structure of the *KNOX1* and *GRF* genes of *N. tabacum* and *N. sylvestris* were obtained from the NCBI database. The respective primers for gene transcripts were selected by the online service NCBI Primer-BLAST and synthesized by LLC Synthol (Tables 1, 2).

Real time PCR was performed in a CFX 96 Real Time System thermocycler (BioRad, United States). 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 for 5 min (DNA polymerase activation), then 45 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 30 s. All PCR runs were performed in 2–3 series and repeated three times. The relative levels of gene transcription were determined

using the respective calibration curves obtained with PCR products formed with primers to the *GaPDh* gene. The efficiency of RT-PCR with primers to the studied genes reached 95–96%.

Preparation of fluorescently labeled peptides. A solution of fluorescein isothiocyanate (FITC) (Sigma, United States) 1 µg/10 µL in 0.5 M sodium bicarbonate was added to the peptide solution in 0.01 M Tris-HCl buffer, pH 7, at a ratio of 1.2 M FITC to 1 M peptide. The reaction mixture was kept at room temperature for 30 min with constant shaking and then applied on a C-18 column and chromatographed on a Bio-Logic DuoFlow (BioRad) chromatograph in acetonitrile gradient (0–100%) containing 1% trifluoroacetic acid.

Analysis of the incorporation of FITC-labeled peptides into the tobacco seedling roots. The FITC marker and the FITC labeled peptides were added to the water at a concentration of 10^{-5} M and incubated with tobacco seedlings for 20 h. Then, root tips with a length of 4–5 mm were cut and fixed in 4% paraformaldehyde (Sigma-Aldrich, United States), prepared on 0.1 M phosphate buffer (PBS), pH 7.2 (Sigma), for 1.5 h at room temperature. After washing three times in PBS, the roots were placed on slides and enclosed in the Moviol 488 medium (Calbiochem, United States). The fluorescence of the preparations was analyzed at 490 nm using $\times 10$ and $\times 20$ lenses of an Olympus BX51 microscope (Olympus, Japan). Images were obtained using a digital camera ColorVien (Germany).

The equipment of the Center for Collective Use of the All-Russia Research Institute of Agricultural Biotechnology was used in this study.

RESULTS AND DISCUSSION

GlyGly, GlyAsp, or Gly in a medium with a concentration of 10^{-7} M significantly stimulate the growth and development of tobacco calli (Figs. 1, 2). An increase in the peptide concentration to 10^{-6} – 10^{-5} M, as well as a 10- to 100-fold decrease in concentration, was accompanied by a decrease in the yield of the tobacco callus fresh weight and the number of regenerants per explant (Table 3). For further study, a 10^{-7} M concentration of peptides and glycine was chosen. Calli grown on media with peptides (GlyGly and GlyAsp) were characterized by a higher fresh weight than the control (Fig. 2, Table 4). Along with the increase in the callus weight, the peptides and glycine increased callusogenesis and leaf formation (Fig. 1). The formation of leaves in tobacco regenerants grown in the presence of peptides started on the 11th–12th day of growing, in contrast to the control callus, where the formation of leaves was observed only on the 14th–15th day. All these data indicate that GlyGly, GlyAsp, and Gly affect growth and cell differentiation and are involved in the plant morphogenesis. In the control, on 28th day, areas of loose and dense morphogenic

callus, as well as individual large morphogenic zones containing a lot of small regenerants, were formed (Figs. 1a–1e). The addition of peptides to the medium increased the total number of regenerants per explant. The most active callusogenesis was observed on media with the GlyGly peptide. In the presence of glycine, the total number of regenerants per explant was also higher; regenerants had a larger leaf area than the leaves of the control plants. In the presence of GlyGly and GlyAsp, the number of regenerants in comparison with the control also increased. The efficiency of regeneration increased by 30–60%, depending on the peptide added (Table 4). The formation of large regenerants with a large leaf area was observed on the medium with Gly and GlyAsp (Fig. 1, Table 4), and in the presence of GlyAsp, the sizes of leaves of regenerants decreased even in comparison with the control.

The significant biological activity of peptides and glycine with a low concentration in the medium (10^{-7} M) indicates that they probably perform a certain regulatory signaling function in the cell and, according to the active concentration in the medium, are quite comparable with known phytohormones.

The investigation of the accumulation of FITC-labeled peptides in the cells of the tobacco seedling roots revealed fluorescence in the apical zone of the root cap and in the epidermal root cells. At the same time, autofluorescence was absent in the tobacco plant roots incubated without FITC or FITC-labeled peptides (Figs. 3a–3c). The incubation of the tobacco roots with FITC-labeled peptides at a concentration of 10^{-5} to 10^{-7} was carried out for 6, 12, and 20 h. The most significant fluorescence in the roots was detected after incubation with fluorescently labeled peptides at a concentration of 10^{-5} M for 20 h. The viability of cells was determined by the differential method of cell visualization with SYTO 9 dyes (Invitrogen, United States) and propidium iodide (Sigma). After 20 h of incubation of tobacco seedling roots in solutions labeled with FITC GlyGly, Gly, and GlyAsp, fluorescence was detected in the cell walls, cytoplasm, and nucleus (Figs. 3d–3o). After incubation of the roots with FITC-GlyAsp (Figs. 3m–3o), the cell walls, cytoplasm, and nuclei had more intensive fluorescence than in the FITC-GlyGly experiment. This means that the peptides and glycine penetrate into the plant cell and its individual compartments, including the nucleus. A similar picture was observed earlier after the incubation of HeLa cells in the presence of different short peptides (Fedoreeva et al., 2011). Especially intense fluorescence in the nucleus was detected in some cells of tobacco roots (Figs. 3i, 3l, 3o). Consequently, the peptides studied were able not only to penetrate into the nuclei, but probably also accumulate in them to a considerable extent in comparison with the cytoplasm. On the way to the nucleus and other cell compartments, peptides can potentially interact with different structures and components of

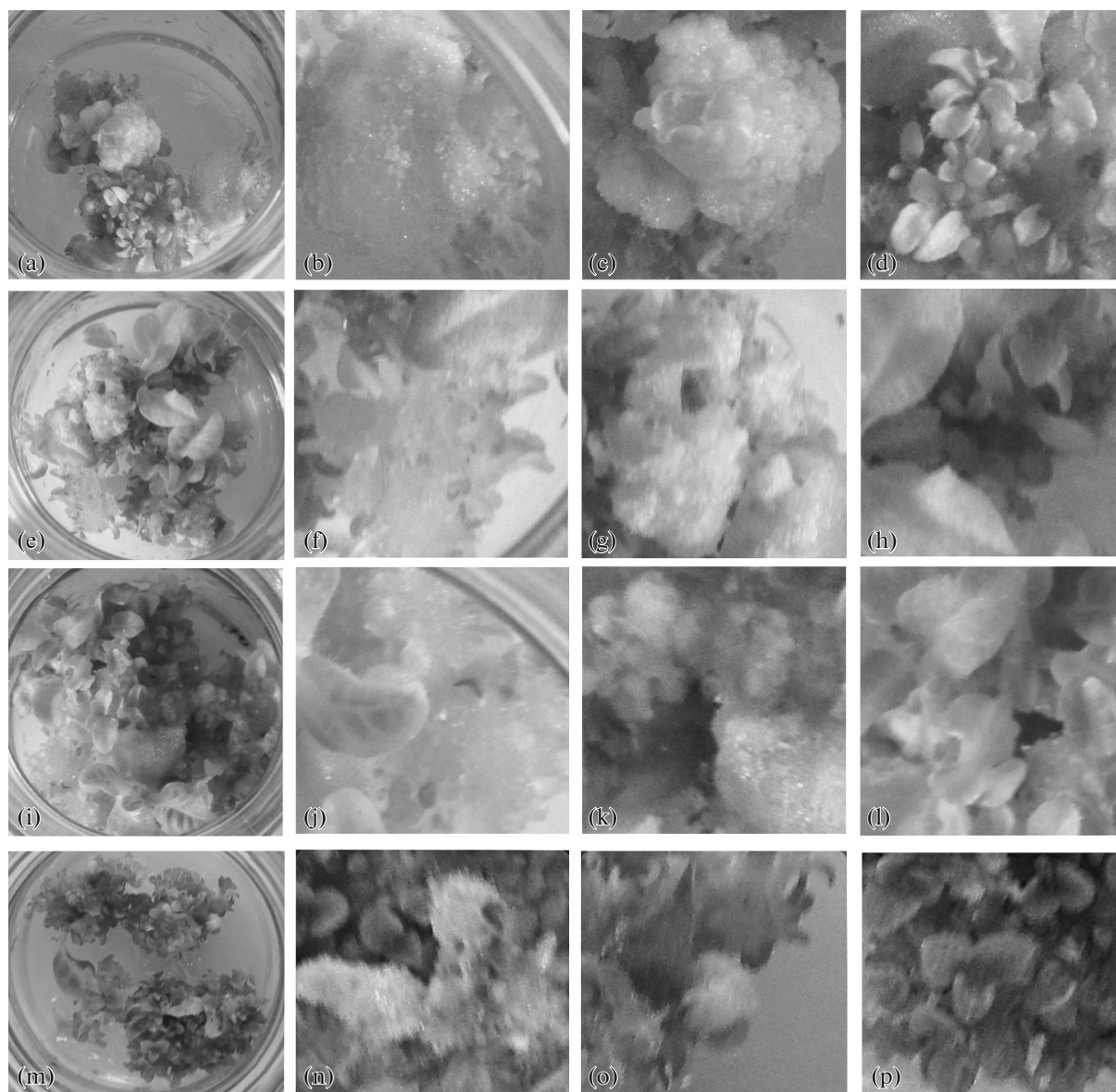


Fig. 1. 28-Day in vitro culture of *Nicotiana tabacum* grown on the MS medium containing 6-benzylaminopurine and indoly-lactic acid. (a–d) Control; (e–h) glycine; (i–l) glycylglycine; (m–p) glycylasspartic acid. (b, f, j, n) Loose morphogenic callus; (c, g, k, o) dense morphogenic callus; (d, h, l, p) regenerants.

the cytoplasm and nucleus, including different proteins, RNA, and DNA. However, the interaction of short peptides with mitochondria and plastids is still unknown. According to our data, short peptides in vitro are capable of site-specific interaction with deoxyribonucleotides, DNA (Fedoreeva et al., 2011), and histones (Fedoreeva et al., 2013). Copper complexes of the GlyGly peptide in vitro bind to the DNA minor groove (Fu et al., 2014).

After incubating the roots with fluorescently labeled peptides, the fluorescence intensities in the different cells of the root were different (Fig. 3). In certain areas of the root, cells devoid of fluorescence were detected. Consequently, the appearance and accumu-

lation of the peptides in the root is to a certain extent cell- and tissue-specific. Most likely, this is associated with the different competence of the cells of different root zones to the interaction, penetration, and accumulation of peptides. Determination of the localization of peptides can be used for rapid assessment of the physiological status of root cells of plants grown under different conditions and the influence of various factors.

The detection of physiological activity in the peptides (induction of cell differentiation and formation, stimulation of callus growth, leaf growth) led us to study the expression of some *KNOX* and *GRF* family genes, which encode transcription factors and are responsible for the actual cell differentiation.

Proteins of the KNOTTED1-like homeodomain (KNOX) family are regulators of stem cell homeostasis in plant seedlings. *KNOX* genes encode transcription factors involved in the arrest of cell differentiation in the apical zone of the seedlings; they have been identified in all monocotyledonous and dicotyledonous plants (Srinivasan et al., 2011). Ectopic expression of *KNOX* genes in various plants leads to dramatic changes in the morphology of leaves and flowers. These changes are also accompanied by a change in the level of hormones. Activation of *KNOX* genes in stem cell homeostasis is closely related to several hormonal pathways in plants (Wenjin and Rongming, 2014).

The *KNAT1*, *KNAT2*, *KNAT3*, *KNAT6*, *LET6*, and *LET12* genes belonging to the *KNOX* class genes (Table 2) have a variety of activity in the organogenesis of stem cells. All *KNOX* genes in *Arabidopsis* (Wenjin and Rongming, 2014) regulate the formation of leaf cells.

GlyGly and GlyAsp, as well as free Gly, affect the expression of the genes of the *KNOX* family (Fig. 4a). In the presence of GlyGly and Gly, the expression of the *KNAT1* gene is significantly reduced, especially in the presence of GlyGly. However, the addition of GlyAsp to the culture medium resulted in a significant increase in the expression of the *KNAT1* gene. The level of expression of the *KNAT2* and *LET6* genes barely changed. The level of expression of the *KNAT3* and *KNAT6* genes depends significantly on the pres-

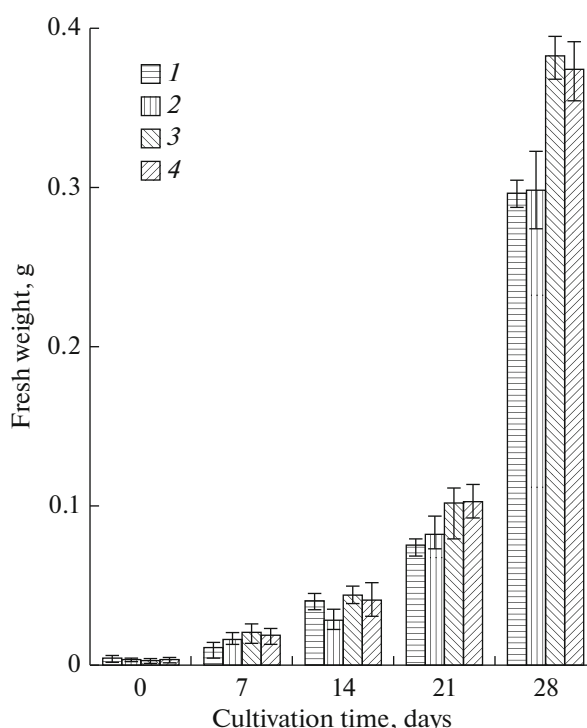


Fig. 2. Dynamics of accumulation of the fresh weight of tobacco (*Nicotiana tabacum*) calli grown on media in the absence and presence of 10^{-7} M glycine and peptides. 1, Control; 2, glycine; 3, glycylglycine; 4, glycylaspartic acid.

Table 3. The efficiency of callus formation and the regeneration potential of *Nicotiana tabacum*, depending on the concentration of dipeptides and glycine in the medium after 28 days of cultivation

| Amino acid/peptide | Concentration in the medium, M | Fresh weight of tobacco callus, mg | Number of regenerants per explant |
|--------------------|--------------------------------|------------------------------------|-----------------------------------|
| Gly | 10^{-5} | 273 ± 13 | 10.6 ± 0.5 |
| | 10^{-6} | 290 ± 14 | 10.7 ± 0.5 |
| | 10^{-7} | 298 ± 12 | 14.4 ± 0.5 |
| | 10^{-8} | 297 ± 15 | 12.9 ± 0.3 |
| | 10^{-9} | 292 ± 16 | 12.6 ± 0.3 |
| GlyGly | 10^{-5} | 304 ± 10 | 10.2 ± 0.3 |
| | 10^{-6} | 348 ± 16 | 10.2 ± 0.7 |
| | 10^{-7} | 383 ± 2 | 13.5 ± 0.4 |
| | 10^{-8} | 379 ± 16 | 12 ± 0.4 |
| | 10^{-9} | 365 ± 14 | 12.1 ± 0.5 |
| GlyAsp | 10^{-5} | 315 ± 15 | 10 ± 0.6 |
| | 10^{-6} | 366 ± 9 | 10.6 ± 0.6 |
| | 10^{-7} | 374 ± 8 | 16.3 ± 0.5 |
| | 10^{-8} | 343 ± 10 | 12.3 ± 0.6 |
| | 10^{-9} | 321 ± 15 | 12.1 ± 0.5 |

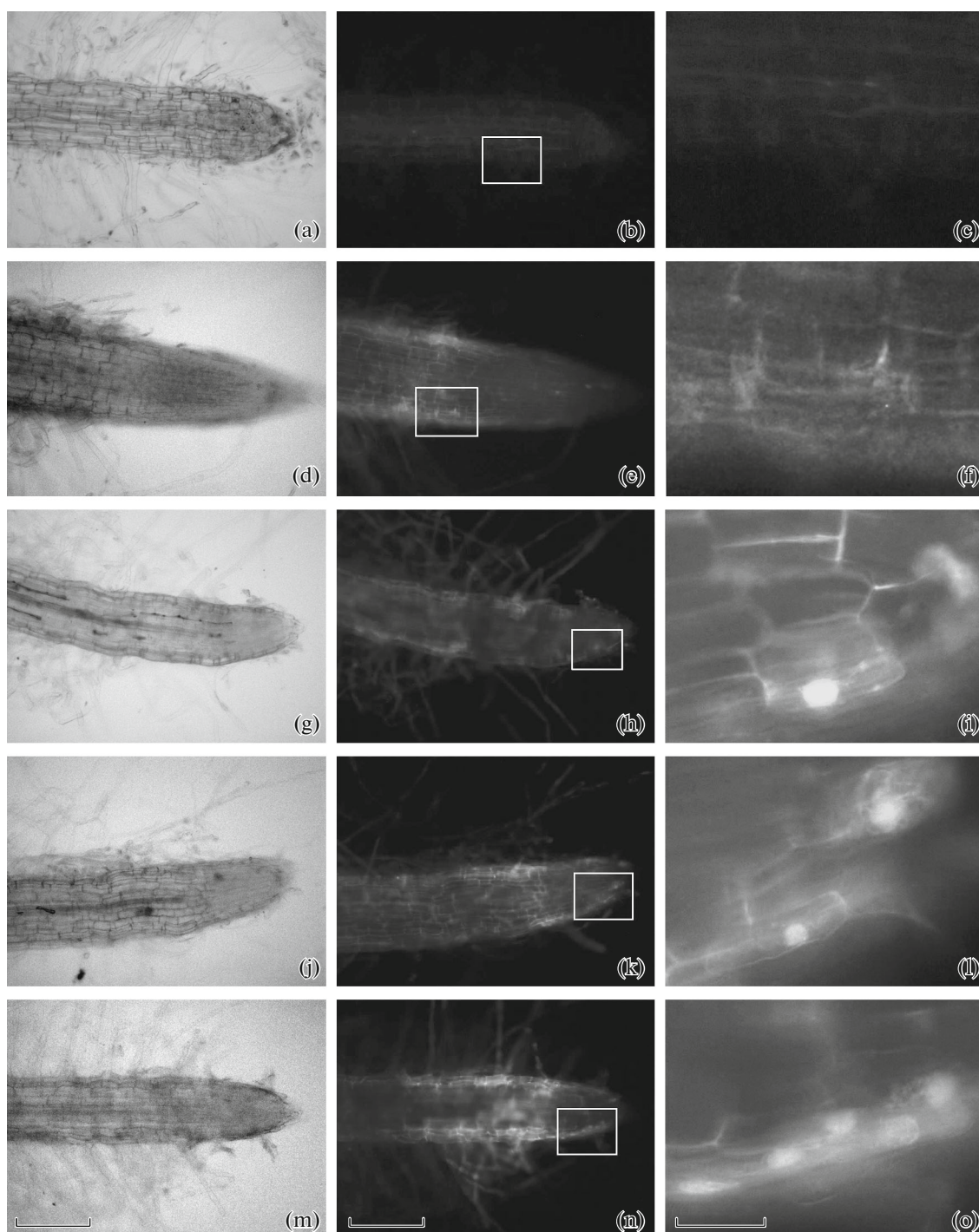


Fig. 3. Visualization of fluorescent markers in the roots of tobacco seedlings. (a, b, c) Without a fluorescent marker (control); (d, e, f) the roots of seedlings after incubation with FITC; (g, h, i) after incubation with FITC-labeled Gly; (j, k, l) after incubation with FITC-labeled GlyGly; (m, n, o) after incubation with FITC-labeled GlyAsp. The frames indicate areas with 10× magnification (scale 20 and 200 μm).

ence of peptides in the medium, and under the effect of these compounds, it can increase by 3–5 times or more in comparison with the control.

Growth-regulating factors (GRF) in plants are specific transcription factors. They play a leading role in the formation of the stem, leaves, flowers, seeds, the

development of roots and the coordination of growth processes in unfavorable environmental conditions (Omidbakhshfar et al., 2015).

Peptides practically did not affect the expression of the *GRF2* gene, but increased the expression of the *GRF1*, *GRF3*, and *GRF4* genes (Fig. 4b). GlyGly and

participated in interaction with primary nucleic acids and already could act as regulators of different biochemical processes. Most likely, modern eukaryotic cells retained this memory and therefore they physiologically and phenotypically respond to the effect of exogenous short peptides. The list of such biologically active exogenous peptides can be quite large, and among them there may be even more active effectors.

Thus, similarly to other short peptides (Fedoryeva et al., 2017), the studied dipeptides GlyGly and GlyAsp and amino acid Gly have significant physiological activity and can be attributed as being effective regulators of plant growth and development.

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REFERENCES

- Fedoreyeva, L.I., Kireev, I.I., Khavinson, V.Kh., and Vanyushin, B.F., Penetration of short fluorescence-labeled peptides into the nucleus in HeLa cells and in vitro specific interaction of the peptides with deoxyribooligonucleotides and DNA, *Biochemistry* (Moscow), 2011, vol. 76, pp. 1210–1219.
- Fedoreyeva, L.I., Smirnova, T.A., Kolomijtseva, G.Ya., Khavinson, V.Kh., and Vanyushin, B.F., Interaction of short peptides with FITC-labeled wheat histones and their complexes with deoxyribooligonucleotides, *Biochemistry* (Moscow), 2013, vol. 78, pp. 166–175.
- Fedoreyeva, L.I., Dilovarova, T.A., Ashapkin, V.V., Martirosyan, Yu.Ts., Khavinson, V.Kh., Kharchenko, P.N., and Vanyushin, B.F., Short exogenous peptides regulate expression of *CLE*, *KNOX1*, and *GRF* family genes in *Nicotiana tabacum*, *Biochemistry* (Moscow), 2017, vol. 82, pp. 521–528.
- Fu, X.B., Liu, D.D., Lin, Y., Hu, W., Mao, Z.W., and Le, X.Y., Water-soluble DNA minor groove binders as potential chemotherapeutic agents: synthesis, characterization, DNA binding and cleavage, antioxidation, cytotoxicity and HSA interactions, *Dalton Trans.*, 2014, vol. 43, pp. 8721–8737.
- Khavinson, V.Kh. and Malinin, V.V., *Gerontological Aspects of Genome Peptide Regulation*, Basel: Switzerland: Karger AG, 2005.
- Khavinson, V.Kh., Fedoreyeva, L.I., and Vanyushin, B.F., Short peptides modulate the effect of endonucleases of wheat seedling, *Dokl. Biochem. Biophys.*, 2011, vol. 437, pp. 64–67.
- Omidbakhshfar, M.A., Proost, S., Fujikura, U., and Mueller-Roeber, B., Growth-Regulating Factors (GRFs): A small transcription factor family with important functions in plant biology, *Mol. Plant*, 2015, vol. 8, pp. 998–1010.
- Srinivasan, C., Liu, Z., and Scorza, R., Ectopic expression of class *1KNOX* genes induce adventitious shoot regeneration and alter growth and development of tobacco (*Nicotiana tabacum* L.) and European plum (*Prunus domestica* L.), *Plant Cell Repts.*, 2011, vol. 30, pp. 655–664.
- Vanyushin, B.F., Ashapkin, V.V., and Aleksandrushkina, N.I., Regulatory peptides in plants, *Biochemistry* (Moscow), 2017, vol. 82, pp. 89–94.
- Wenjin Zhang and Rongming Yu, Molecular mechanism of stem cells in *Arabidopsis thaliana*, *Pharmacogn. Rev.*, 2014, vol. 8, no. 16, pp. 105–112.

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