Peptide regulation of plant and animal morphogenesis:
general mechanisms and specificity of action

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Abstract. Regulatory peptides are one of the main signaling molecules that provide intercellular interactions in biological systems. Thus, regulatory peptides are known as morphogens - biologically active substances involved in the regulation of the morphogenesis of animals and plants. Until now, the interaction of plant and animal peptides and, in particular, the ‘cross’ effect of plant peptides on the morphogenesis of animals and vice versa has not been sufficiently investigated.

The present work is devoted to the study of the effect of growth regulator peptide (CLV 3) of Arabidopsis on the morphogenesis of animals - freshwater flatworms – planaria. This peptide is an effective regulator of growth and differentiation of plant roots in low and ultra-low concentrations.

Various biophysical methods have shown that this plant peptide at a concentration of $10^{-9}$M is also an effective morphogen of animals. At the same time, the specific action of the peptide on ‘unrelated’ biological targets has been found. The peptide CLV 3 stimulates on the morphogenesis of plant roots in concentrations up to $10^{-12}$M, while for planarian regeneration it acts only at concentrations up to $10^{-9}$M, at which it does not show a noticeable effect on root plant growth. When this peptide was applied to the of stem human stem cells Th culture, there was no effect observed.

The results of the study confirm the thesis that there is a general biological level and peptide regulation mechanisms that are similar for animals and plants, and also reveal the nature of the specifics of the ‘direct’ and ‘cross’ action of this mechanism.

1. Introduction
The purpose of this work is to study the effect of the biologically active plant dodecapeptide CLV3 on the process of regeneration of flatworms – planaria as an example of the possible influence of peptide systems of plant regulation on the processes of animal morphogenesis.
Morphogenetically active peptides can be an important tool for molecular interaction between representatives of different biological taxa living in close proximity. An example of direct contact of plants and animals can serve as flat worms and plants in the roots of which they inhabit.

Planarian regeneration is regulated by various biologically active peptides of animals, being morphogenic, therefore flatworms were used as a regenerative model for the study of morphogenesis. It has been shown that different peptides are able to control the processes of proliferation and regeneration in extremely low concentrations (up to $10^{-15}$M), which allows us to consider peptides-morphogenes as one of the finest instruments of molecular control [1−4].

The peptide pCLV3 is expressed in the stem portion of Arabidopsis thaliana, influencing the growth and development of the terrestrial part and plant roots in concentrations up to $10^{-9}$M – $10^{-12}$M [5].

In this paper, the effect of this peptide on morphogenesis was studied during regeneration of the cephalic end of the body of planar worms during five days of regeneration using non-invasive biophysical recording methods. During the first 24 hr after the cutting, ultra-weak photon emission (SWFE) was recorded in the planarians, and after 72 hr and after 120 hr of regeneration the epimorphic growth of the head end (blastema) and the rest of the planarian body were recorded by the method of IDM [6, 7].

2. Materials and methods
The work was carried out on an asexual laboratory race of the planaria Girardia tigrina, which were kept in room conditions at a temperature of 20 – 21 °C. The animals were fed weekly with Diptera larvae. Before the experiments, the planarians had starved for a week.

The dodecapeptide CLV3, having the following sequence of amino acid residues RTVPSGPDPPLHH, was provided by the Laboratory of Peptide Chemistry of the Institute of Bioorganic Chemistry RAS. MM Shemyakin and YuA Ovchinnikov. The dodecapeptide CLV3 was prepared by N-α-FMOC solid phase synthesis and purified by HPLC using a standard procedure. The identity and purity of the synthesized peptide at the level of 95% is confirmed by reversed-phase HPLC, and also by means of MALDI-TOF/TOF mass spectrometry.

2.1. Superweak photon emission (SWFE)
The registration of SFE was carried out with the help of the Biotox 7a luminometer. The sample is equipped with a 9750QB/1 photomultiplier (9750QB/1, EMI Electronics LTD, Middlesex, UK) with a spectral sensitivity region of 380-710 nm, with a maximum in the blue-green region, 420-500 nm. The measurements were carried out at a temperature of 21.0 ± 0.2 °C, in the photocount counting mode with an interval of 1c. Further analysis of the results was carried out according to the previously described method [7].

A group of 30 planarians immediately after decapitation was placed in a scintillation counting bottle in 10 ml of an aqueous solution of lucigenin ($10^{-9}$M), and the registration of SSE was started. In special experiments, the registration of SWFE in planarians in the control and experimental groups was carried out in parallel on two Biotox-7a luminometers.

2.2. Intravital digital morphometry (IDM)
The method is based on the registration of a photocontrast between the old (pigmented) and new (uncoated pigment) parts of the body of the regenerating planaria. To obtain the planar images, a Stemi 2000C binocular computer microscope equipped with an AxioCam MRc (Zeiss) video camera was used. Regeneration was caused by amputation of the head part of the planar body and the regeneration was recorded at 72 and 120 hrs after transection.

The digitized images of the regenerating planarians were processed in the Plana 5.0 program, which allows analysis of electronic images of the planaria and determination of the areas of the projection of the regenerant body and blastema. As a criterion for regeneration, the ratio of the area of the blastema to the total area of planaria was applied on the certain hours of regeneration [6].
2.3. Definition of the mitotic index (MI)
The mitotic index during regeneration was determined by counting the mitotic figures in the suspension of planar cells [8]. The number of metaphases per 1000 cells (mitotic index) was counted within 24 hours after transection at colchicine exposure. To visualize the mitotic figures, Hoechst 33342 dye was used. Metaphases were recorded with the AxioVert 200M (Zeiss) fluorescent microscope.

2.4. Cytotoxicity assay
Cytotoxicity was analyzed using the postnatal dental pulp stem cells Th (DPSC) [9]. For the experiment, DPSC were plated in wells of a 96 well plate at a concentration of 25,000 cells/cm² in DMEM/F12 medium containing 5% FBS serum. After 18 hours, the medium was removed and 10⁻⁵-10⁻¹¹M peptide solution in DMEM/F12 medium containing no serum attachment factors was added. As control of the passage of the MTT-test, 10% dimethyl sulfoxide (DMSO) was added to the cell culture medium. As a general control, cells cultured in DMEM / F12 medium containing no serum attachment factors were used. In the second case, the medium with the peptide was changed every 24 hr.

To evaluate the proliferation, death of human stem cells and the of the material, a MTT test was used based on the direct correlation of the number of living cells and the intensity of the metabolism of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) insoluble dark-colored formazan soluble in dimethylsulfoxide under the action of dehydrogenases [10].

2.5. Statistical analysis
The statistical analysis of the results of the mitotic index determination was carried out using the Mann-Whitney criterion for small groups. The results of the IGM image analysis were carried out using the SygmaPlot 9.0 software package. The mean value, the mean error, the confidence interval for the significance level of 0.95 was determined. The comparison of the averages was carried out using the Student's t-test.

3. Results and discussion
In this work, two non-invasive biophysical approaches were used to register the regeneration processes. In the first 24 hr after the transection, we recorded own planarian superweak photon emission (SWFE), and then on the same group of animals – epigenetic regeneration was recorded 72 and 120 hr after decapitation by IDM. In our study we used the registration of SWFE as a remote test for the lifetime detection of ROS as a regulator of cell proliferation and their differentiation, as well as an indicator of metabolic activity during regeneration, within the first 24 hr after transection.

During regeneration, in many animals, including planarians, the level of generation of ROS increases [11]. This process of sequential four-fold one-electron reduction of oxygen is accompanied by generation of electron excitation energy and leads to emission of photons of super-weak intensity which is detected by highly sensitive photomultipliers. In our study we used the registration of SWFE as a remote test for the lifelong detection of ROS.

After operations, all fragments of control and experimental animals were placed in a luminometer for 24 hr. Further, the blastema of the decapitated planaria of the experimental and control groups was recorded at 72 and 120 hr after the operation by the IDM method.

In a series of the very first test experiments on the study of the biological activity of CLE-peptides, dodecapeptides were synthesized, based on the genomic data on the sequence of amino acids in the region of the conserved domain of 26 representatives of the CLE A. thaliana family [12].

In this study, we used one of the most studied and characterized CLE peptides of A. thaliana the dodecapeptide CLV3. It was previously established that when the peptide CLV3 is added to the culture medium at concentrations of 10⁻⁶M to 10⁻⁸M, the growth of the main root of the Arabidopsis seedlings is significantly suppressed, and at the same time the formation of lateral roots is stimulated. At a concentration of 10⁻¹²M peptide, CLV3 causes stimulation of the growth of the main root. At this concentration dodecapeptide CLV3 reduces the number of lateral roots [5].
The study of SWFE was carried out in parallel in two groups (experimental and control) of 30 decapitated animals on two Biotox-7a luminometers. The results are shown in Figure 1. Immediately after the cutting, an intensive burst of SWFE is observed. Then, within one to two hours after the transection, the relaxation of SWFE is registered to the level of the intact planaria [7].

The CLV3 peptide in the first two hours after the decapitation operation accelerated the relaxation of the level of SWFE twice as compared with the control group. The main peak of SWFE was observed 12 hours after the decapitation operation: the level of SWFE was twice as high under the action of the peptide, which was confirmed in 5 series of experiments. This peak coincides in time with the peak of proliferation of stem cells of planarians – neoblast [13]. This assumption was confirmed in experiments on the study of the mitotic index of planaria during the first day of regeneration.

![Figure 1](image)

**Figure 1.** Effect of the peptide CLV3 on the superweak photon emission (SWFE), of the planaria *G. tigrina* during the 24 hr after decapitation. Abscissa is the time of regeneration in hours. Y-axis is the intensity SWFE in photons per second. 1 – dynamics of SWFE during of regeneration under the action of peptide CLV3 (10⁻⁹M). 2 – dynamics of SWFE during of regeneration in the control group.

The peptide CLV3 (10⁻⁹M) stimulated cell proliferation, on average, by 17% compared to the control. Further study of the regeneration of these planar groups at 72 and 120 hr after transection by IDM also revealed the effect of stimulation of planar regeneration by the CLV3 peptide in five series of experiments.

The CLV3 peptide at a concentration of 10⁻⁹M accelerates the regeneration of the planaria by 1.3 times at 72 hr and by 1.2 times after 120 hr after the operation compared to the control samples (Figure 2).

However, unlike the concentration of 10⁻⁹ M, the study of the stimulating effect of the peptide at concentrations of 10⁻¹² M, 10⁻¹⁴ M and 10⁻¹⁸ M did not significantly differentiate the experimental samples from the control ones. Also, the effect of the peptide on the viability of mesenchymal stem cells *Th* from the human tooth pulp was not detected. This result was obtained both in the cultivation of cells without a change in the medium, and in a daily change in the medium and in applying a fresh dose of the peptide during the 3 days of the experiment.
Figure 2. The action of the peptide on the regeneration of the planaria on the 3rd and 5th day of regeneration in comparison with the control group. On the y-axis – the regeneration criterion in percent.

Thus, in our experiments, the specificity of direct and cross-reactivity of regulatory peptides on plant, animal and human cells were revealed. Under the action of animal peptides on the morphogenesis of animals and plant peptides on the morphogenesis of plants, effects of superweak concentrations were obtained: up to $10^{-15}$ M for luteinizing hormone releasing hormone (LHRH) [1,3] and up to $10^{-12}$ M for peptide plants pCLV3 [5]. When the plant peptide cross-reacts to the cells of animals and humans, it acts only at a concentration of $10^{-9}$M on the regeneration of the planaria or does not at all affect the survival of human stem cells.

It is interesting to study whether there is the same specificity of ‘direct’ and ‘cross’ action for other peptides of animals and plants on other plant and animal test systems.

The molecular mechanisms of biological processes are similar for both plant and animal biosystems. Plants and animals constantly interact with each other at the macro level through symbiosis, parasitism, and mimicry. The same processes take place at the micro level, as well, in particular, at the level of conservative cellular processes, such as proliferation and differentiation of cells, which underlie the growth and morphogenesis of both plants and animals.

However, unified (general) mechanisms of regulation of the morphogenesis of animals and plants at the epigenetic level have not been studied enough. This also applies to the mechanisms of peptide regulation of cell proliferation and differentiation.

An example of such a cross-regulating system is the detection of receptors for plant peptides of the CLE group in parasitic flatworms [14]. Expanding the understanding of the role of this regulatory system for general mechanisms of plant and animal morphogenesis will make it possible to clarify, among other things, the unification and specificity of these molecular instruments as a universal system of peptide regulation in living systems.

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