REVIEWER REPORT

on a competition for the academic position "Associate Professor" in a professional field 4.3. Biological Sciences, scientific specialty "Biochemistry" announced in SG № 17/26.02.2021 for the needs of the laboratory "Regulation of gene expression" of the Institute of Plant Physiology and Genetics - Bulgarian Academy of Sciences (IPPG-BAS)
Candidate: **Kiril Mihailov Mishev**, PhD, Assistant Professor in the Laboratory "Regulation of Gene Expression" of IPPG-BAS
Reviewer: Professor Liliana Georgieva Gigova, PhD, IPPG-BAS

The only candidate in the announced competition, Assistant Professor Kiril Mishev, has provided all the documents required.

Career and thematic profile of the candidate

Kiril Mishev graduated from the Faculty of Biology at Sofia University "St. Kliment Ohridski" in 2004 with a Master degree in Plant Physiology. In the period 2005-2009 he was a doctoral student at the Institute of Plant Physiology "Acad. M. Popov" (currently IPPG), and in the beginning of 2010 acquired the educational and scientific degree "Doctor" in the scientific specialty "Plant Physiology" after defending a PhD thesis: "Functional state of the photosynthetic apparatus and gene expression in chloroplasts in dark-induced and natural senescence". In 2008, he was elected as an Assistant Professor in the scientific specialty „Biochemistry" at the IPPG. The research activity of Dr. Kiril Mishev in the field of plant physiology, cell biology and biochemistry is focused on the functional organization of the photosynthetic apparatus, regulation of chloroplast genes expression, leaf senescence, phytohormonal signaling pathways, intracellular membrane traffic. In the period 2007-2019 he specialized in leading scientific institutions in Germany, Belgium and the Czech Republic, which has contributed to the enrichment of his knowledge, skills and competencies in the application of various research techniques.

Research metrics

The total number of research papers of Dr. Mishev is 24 (**Q1 - 16** publications; Q2 - 2; Q4 - 2, publications in peer-reviewed journals, not indexed in Web of Science and Scopus - 4; total JCR IF: **104.409**); the total number of citations is **338** as **285** are WoS/Scopus; his h-index is **8** (Scopus and WoS). For participation in this competition, 16 scientific publications are presented, outside the habilitation thesis (**14** with **Q1** and 2 with Q4; total JCR **IF: 98.964**), in 4 of them K. Mishev is the first author (3 with Q1 and 1 with Q4). It is impressive that the publications presented for the competition are mainly in authoritative international journals of very high rank, such as Nature Chemical Biology (IF 12.587); Nature Communications (IF 12.124); Proc Natl Acad Sci USA (IF 9.580); The Plant Cell (IF 8.631); Current Opinion in Plant Biology (IF 7.848); Chemistry & Biology (IF 6.586); Plant Physiology

(IF 6.456). The high level of the scientific production of Assistant Professor Mishev confirms the significance of his research and the results obtained, as well as the wide interest and recognition by the scientific community.

Candidate's contribution to collective publications

The publications submitted for the competition are co-authored with Bulgarian and/or foreign scientists (mainly from Belgium, USA, Czech Republic and Germany). Dr. Mishev's involvement in idea generation (he is the first author of 4 of the publications), in conducting experiments, visualization, analysis and interpretation of the results is significant, as evidenced by the detailed description of his personal contribution to each publication.

Participation in scientific forums and research projects

A list of 23 participations of Kiril Mishev in scientific forums in Bulgaria and abroad with 15 posters and 8 oral presentations is provided. In 7 of the oral reports Mishev is the first author. The total number of his participations in research projects is 18 (9 national and 9 international), in 4 of them he is the project leader/coordinator.

Teaching and training activities

Assistant Professor Mishev has conducted practical training of 8 students from the Faculty of Biology at Sofia University "St. Kliment Ohridski" under 2 projects "Student Internships" within the Operational Program "Human Resources Development" for a total of 1860 hours in 2013, 2020 and 2021. In the academic year 2014-2015 he co-supervised a thesis of a student from HoGent, Belgium on "Plant growth modulation through chemical genetics".

Research profile and main scientific contributions

The research activity of Dr Kiril Mishev resulted in original papers that are provided for participation in the competition, and cover 4 interrelated and complementary scientific topics with basically fundamental contributions.

The first scientific topic includes research achievements linked to the molecular mechanisms of the regulation of intracellular membrane traffic based on the approaches of chemical genomics and proteomics (publications B4_1, B4_2, Г7_3, B4_4 и Г7_7). This research topic is aimed at identifying and characterizing the mechanisms of action of new low molecular weight chemical compounds that specifically affect the activity of protein trafficking regulators, thus presenting an alternative to classical and genetic tools for elucidating the cellular and developmental roles of these crucial regulators. A growth inhibitor (called Secdin) that causes aberrant accumulation of plasma membrane marker proteins in late endosomal compartments has been identified (G7_3). The targeting of transported proteins to the degradation pathway in the lytic vacuole is not associated

with a direct effects of the inhibitor on the ubiquitination system, but with a delay in the processes of exo- and endocytosis. It has been found that, unlike the classical ARF-GEF inhibitor Brefeldin A (BFA), Secdin can interact with all ARF-GEF proteins (**g**uanine nucleotide **e**xchange **f**actors for **A**DP-**r**ibosylation **f**actor GTPases) of the Arabidopsis proteome, as the interaction is outside the catalytic Sec7 domain of ARF-GEFs. Secdin and BFA affect their target proteins through different mechanisms, making Secdin useful in studies where ARF-GEF-dependent endomembrane transport cannot be manipulated with BFA (BFA-insensitive ARF-GEFs). Another inhibitor of the intracellular membrane traffic, Endosidin 4 (ES4) that differs in chemical structure from Secdin and BFA has been identified (B4_2). ES4 treatment results in disruption of all ARF-GEF-dependent pathways, however, unlike Secdin, this inhibitor interacts selectively with only particular ARF-GEFs in Arabidopsis. The effect of ES4 on ARF-GEFs is manifested by a change in the ratio between the membrane-bound and cytosolic fraction of ARF1 GTFase - a substrate of ARF-GEFs, as the cytosolic, inactive fraction increases. A new inhibitor of clathrin-dependent endocytosis in plant cells has been found, termed Endosidin9 (ES9), which has been shown to be active in other eukaryotic systems (B4_4). ES9 treatment has also non-specific effects, such as a drastic decrease in cell ATP levels resulting from the dissipation of the membrane potential in the mitochondria, as well as a decrease in the pH of the cytoplasm. The inhibitory effect of ES9 has been found to be related to its protonophore activity, which leads to a decrease in cytoplasmic pH. Similar non-specific effects are identified by the authors for the first time in tyrphostinA23 (TyrA23), the most widely used endocytosis inhibitor. By chemical modification, ES9 analogue was obtained - ES9-17, which does not cause a decrease in the pH of the cytoplasm, but has the preserved ability to block endocytosis (B4_1). It has been convincingly evidenced that the mechanism of specific action of ES9 and ES9-17 is related to direct interaction with the heavy chain of the protein clathrin. The results of these studies affirm ES9-17 as the only **specific** inhibitor of clathrin-dependent endocytosis for the use in plant cellular biology. A review article (G7_7) provides a valuable comparative analysis of intracellular vesicular trafficking pathways in different eukaryotic systems. The screening studies published until 2013, and linked to the identification of new low molecular weight traffic effectors, are summarized. The potential of the new compounds for biological activity in more than one eukaryotic system depending on the degree of conservatism of the target proteins is analyzed.

Another important direction in the research activity of Dr. Kiril Mishev with original fundamental contributions is a study of the mechanisms of hormonal regulation in plants and

interaction between phytohormonal signaling pathways (5 publications). New aspects of the regulation of auxin biosynthesis and polar auxin transport integrated with ethylene signals at salt stress conditions have been identified, using different approaches and methods (Γ7_1). Two model systems are used in this study - an ethylene-insensitive *Arabidopsis* mutant and a constitutive mutant with a constantly active ethylene signaling pathway. The negative effect of salt stress is less pronounced in the constitutive mutant. In this line, the ethylene-induced consistently high levels of auxin have been associated with the enhanced expression of enzymes from the biosynthetic pathway of indolyl acetic acid, as well as with the stable expression of auxin transporters. Differences in the effect of salt stress on auxin transport in the root epidermal layer and vascular system in the two studied genetic systems have been found. The results show that upon salinity, ethylene signaling exerts cell type-specific effects on auxin concentration and transport, expressed mainly in the epidermis of the root tip. Another study (Γ7_8) has clarified the significance of receptor-dependent endocytosis for the signaling activity of the brassinosteroid receptor BRI1. A biologically active fluorescently-labeled brassinosteroid (AFCS) has been developed to visualize for the first time endocyt pathway of the receptor-ligand BRI1-AFCS complex in living Arabidopsis cells. The internalization of the BRI1-AFCS complex has been accomplished by clathrin-dependent endocytosis involving ARF-GEFs. Impairment of endocytosis enhances signal transduction by retaining active receptor-ligand complexes at the plasma membrane, whereas accumulation of complexes in the trans-Golgi network does not affect brassinosteroid signaling. Overall, the results show that the BRI1 fraction of the plasma membrane plays a major role in the perception of the hormonal stimulus, while endocytosis weakens the cellular response to the brassinosteroid signal. The role of U-box E3 ubiquitin ligases PUB12 and PUB13 in the brassinosteroid signaling pathway has been defined (B4_3). It has been shown that both enzymes directly ubiquitinate the BRI1 receptor kinase. The interaction between ubiquitin ligases and BRI1 is stimulated by the presence of a hormonal signal (brassinolide) on the cell surface. Activated BRI1 binds to PUB13 by an established from the authors unique mechanism, regulated by phosphorylation, which in turn enhances the activity of PUB13 to ubiquitinate BRI1. Impaired expression of *PUB12* and *PUB13* (in the double *pub12pub13* mutants) results in increased BRI1 protein abundance and plasma membrane residence time, and decreased BRI1 endosomal fraction, thus proving the key role of PUB12/PUB13-mediated ubiquitination in BRI1 endocytosis and intracellular degradation. For the first time in functional plant biology, the induced protein aggregation approach has been applied and demonstrated that the sequence specificity of aggregation-prone peptide regions (APRs) can be

used to selectively attenuate the function of proteins with different localization and function (G7_5). BIN2 kinase, a negative regulator of the brassinosteroid signaling pathway, has been studied as a model protein. Genetic constructs containing nucleotide sequences encoding identified and optimized APRs in BIN2, in a common reading frame with a reporter protein gene (GFP), have been developed. Bimolecular fluorescence complementation (BiFC) assays after temporary expression of these constructs in tobacco leaves, demonstrated specific interactions of APRs with their target proteins, BIN2 and other proteins in the SHAGGY-like kinase family. In transgenic *Arabidopsis thaliana* lines, a constitutive response to a brassinosteroid stimulus has been established. This phenotype is associated with aggregation and attenuation of BIN2 kinase activity. Valuable overview article (G7_6) summarizes the information to 2014 on the available low molecular weight biologically active substances that can be used to study the brassinosteroid (BR) effect. Strategies for the development of new inhibitors of the enzymes from the BRs biosynthetic pathway, as well as specific chemical modulators of the functions of components of the BRs signal transduction chain, are analyzed. The approaches for creating new biologically active synthetic analogues of BRs in the light of the published crystal structure of the extracellular domain of the BRI1-ligand complex are also commented.

The acquired new, valuable knowledge in these two areas could have future **practical application** for the development of innovative biotechnological and agrochemical approaches to improve the growth traits in plants, by modulating intracellular trafficking of hormone receptors and transporters.

The results of Mishev's research on structural and functional aspects of the reaction of the photosynthetic apparatus to adverse environmental conditions such as darkening or chemically-induced changes in the properties of thylakoid membranes (4 publications) have also great fundamental and applied potential. Particular attention is paid to the differences in the ability of cotyledons and true leaves to recover from the applied stress. Differences in the mechanisms of photoprotection in Arabidopsis cotyledons and true leaves in terms of non-photochemical quenching and non-regulated energy dissipation, depending on the type of the applied dark stress locally or at the whole-plant level have been established (G7_11). The transcripts levels of the chloroplast genes *psaB* (encoding the A2 protein from the PS I reaction center) and *rbcL* (encoding the large Rubisco subunit) decreased to approximately the same extent in individually darkened and in cotyledons of the whole darkened plants. In the leaves, however, the decrease in mRNA levels of the two genes upon individual darkening is stronger than in the whole plant darkening, indicating

that, unlike cotyledons, the photosynthetic response of darkened rosette leaves depends on the illumination of the rest of the plant. Expression analyzes in darkened cotyledons and leaves revealed a drastic reduction in mRNA for FtsH5 and Deg1 proteases involved in the repair of damaged PS II complexes, which the authors associated with the lack of photooxidation of PS II proteins under dark conditions. Comparative analyzes of chlorophyll fluorescence and mRNA levels of the chloroplast genes studied and the marker *SAG12* gene (a nuclear gene encoding leaf senescence-associated cysteine protease SAG12) during the recovery phase from the dark provides original data showing the ability of the cotyledon photosynthetic apparatus to overcome the negative effects of dark stress. Upon re-illumination, the individually darkened true leaves show typical chloroplast senescence symptoms, while the true leaves of darkened whole plants maintain a high photosynthetic capacity. A difference has been found in the sensitivity of plastid RNA polymerases PEP and NEP to dark stress in zucchini cotyledons (G7_10). The overall chloroplast transcription rate decreases due to a significant decrease in the activity of chloroplast-encoded PEP polymerase, while nuclear-encoded NEP is not significantly affected by stress. Compared to chloroplasts, the synthesis of total RNA in the cell nucleus is significantly less affected due to the almost unchanged activity of RNA polymerase I. The activity of RNA polymerase II decreases, which is more pronounced in individually darkened than in the cotyledons of whole darkened plants. In the post-stress period, the rate of plastid transcription in the cotyledons of whole darkened plants is three times higher than in naturally senescing controls, which is a clear indication of delayed senescence. A dark-induced decrease in *psaB* and *rbcL* mRNA content has been found, due at least in part to a reduced rate of synthesis, while transcription of the *psbA* gene is not affected. Another study demonstreted that local darkening of cotyledons or primary zucchini leaves affects the senescence of neighboring normally illuminated leaves (G7_9). The compensatory response of non-obscured leaves is analyzed by a combination of biophysical, biochemical and microscopic approaches. The results show that the darkening of the cotyledons causes specific changes in the physiological status, including a decrease in the total cytokinin levels, increased activity of cytokinin oxidase/dehydrogenase, increase in the content of ABA in the adjacent illuminated true leaf, which are considered as signs of stress. The darkening of the first true leaf adjacent to the cotyledons has the opposite effect on these indicators in the illuminated cotyledons. The lack of influence on the photosynthetic activity and the expression of marker chloroplast genes show that the process of photosynthesis is not a primary target in the mechanisms of communication between the two types of shoot organs. **New aspects** of the mechanism of biological action of the amphiphilic peptide

melittin from bee venom have been discovered, using chloroplast thylakoid membranes as a model system (G7_12). Significant differences are found in the effect of melittin on the electrophoretic mobility and light-scattering properties of thylakoids depending on the concentration of the peptide and the salt composition of the medium. These differences have been interpreted in relation to shielding of negative charges from the membrane surface, increasing the permeability of the lipid bilayer and changes in the geometry of the membrane structures. Melittin reduces the primary photochemical efficiency of PS II only when the chloroplasts are incubated with higher concentrations of the peptide, and this negative effect is better expressed at low ionic strength (high degree of membranes stacking) than at high ionic strength.

The fourth research topic is related to the elucidation of the structural and functional organization of ribosomal DNA in Hordeum (publications G7_2 and G7_4, respectively). Unmethylated CCGG regions are located in a small fraction of the rDNA repeats of common barley (*Hordeum vulgare*) near the transcription start site, in the external transcribed spacer, and in the rRNAs coding sequences. A deletion mutant is used as a model system, which has instead of two, only one nuclear organizer, but with increased transcriptional activity. The authors associated the compensatory increased activity with the concomitant increased degree of rRNA hypomethylation, since no changes in the number of *rRNA* genes or in the structure of the intergenic spacer are found in the deletion line. The nucleotide sequence and structural elements in the 25S-18S rDNA region of the genomic DNA of *Hordeum bulbosum*, which has one nuclear organizer, are determined. It has been found that the intergenic spacer contains two regions with sub-repeats R143 (2 repeats) and R128 (5 or 6 repeats), showing a similarity in the nucleotide sequence with R79 and R135, respectively from *H. vulgare*. A sequence of 31 bp in R128/R135 repeats is conservative in all studied cereal species, suggesting its regulatory role in transcription. The transcription initiation site, which is recognized by RNA polymerase I, has been also determined.

CONCLUSION

A thorough review of the materials submitted for the competition shows that the candidate fully meets the requirements of ZRASRB, the Regulations for its implementation and the specific conditions in IPPG-BAS for holding the academic position “Associate Professor”. The high- quality research, the overall impact of the scientific contributions, the high citation rate and the active teaching and project activity characterize Dr. Kiril Mishev as a distinguished, recognized by the international scientific community researcher in plant physiology, cell biology and biochemistry

with excellent theoretical and methodological expertise and teamwork skills. All this gives me a solid basis to unconditionally support his application and to recommend to the respected members of the Scientific Jury and the Scientific Council of IPPG-BAS to support the awarding of Assistant Professor Dr. Kiril Mishev with the academic position “Associate Professor” in Biochemistry.

18.06.2021

Reviewer:
/Prof. Liliana Gigova, PhD/