Closing report- Short presentation of achievements made in the whole duration of the NKFIH K120638 project- 01.10.2016-30.11.2019

Report is made in accordance to the original research project proposed.

Fulfillment of the tasks proposed

Task 1- the study of cytoskeletal (microtubule/ MT) alterations induced by ROS and the counteracting effects of ROS scavengers. ROS inducer chemicals (that do not inhibit protein phosphatases) and scavengers will be used to complete this task.

This task has been completed in the first year of the project. Mainly, the ROS inducer diquat (DQ) and the scavenger Trolox (α-tocopherol was also checked) was used for this purpose. We have detected MT alterations both in mitotic and non-mitotic cells. Model plants were Arabidopsis (Col0) and *Nicotiana tabacum* SR1. The publication concerning this task is under preparation.

Task 2- the study of common cellular alterations induced by both protein phosphatase inhibitors and ROS. We will compare PP1/ PP2A inhibitors to ROS inducer chemicals (that do not inhibit protein phosphatases) to complete this task.

Completion of this task has been started in the first year of the project and now completed. Mainly microcystin-LR (MCY-LR) was used as the protein phosphatase inhibitor. We have concentrated on mitotic spindle organization, but cytoskeleton and chromatin of non-mitotic root cells was also studied. We have proven that Arabidopsis seedlings are very sensitive to oxidative stress. Thus, treatment with ROS inducers is inducing severe mitotic cytoskeletal alterations. The use of model plants with different oxidative stress tolerance did enable us to develop an "atlas" of mitotic and generally, cellular alterations induced by ROS and protein phosphatase inhibition, respectively and enabled us to compare the cytological effects of these two factors.

During our studies, we have demonstrated that MCY-LR as well as CYN- another drug we have proven to be a protein phosphatase inhibitor- induce mitotic and non-mitotic MT alterations in *Vicia faba* and generally, in vascular plants (Garda et al., 2016; Máthé et al., 2016, 2017; M-Hamvas et al., 2017, the latter two related directly to this project). In the second year, we completed these studies, by showing that allyl-isothiocyanate (AITC), a volatile oil from horseradish, has several significant effect both in vitro (on purified catalytic subunits) and in vivo (in roots of *Vicia faba*) on the protein phosphatases PP1 and PP2A and amplifies the effects of MCY-LR on PP1/PP2A and on subcellular organization during mitosis. The most interesting effect of the use of a MCY-LR + AITC combination is metaphase arrest. An exciting future direction is to investigate whether similar effects are exerted on mammalian cells, because if this is so, such a combination of inhibitors could have the potential to be used as a chemotherapeutic agent in tumor therapy. Meanwhile, AITC does not induce oxidative stress and besides MCY-LR it is an ideal tool to study the effects of PP1/PP2A on the

regulation of mitosis. This work allowed us to publish a manuscript to Frontiers in Plant Science, a leading journal of the field (Garda et al., 2018, see the list of publications prepared in the second year of project at the end of this report).

Overall, the use of known ROS inducers (DQ) on one hand and biologically active compounds (AITC, MCY-LR) on the other hand allowed us to complete experimental work for Task 2 and now we are preparing a further manuscript related to this subject. Moreover, we have published a manuscript regarding the cytological effects of MCYs on aquatic plants in the real environment (a case study of co-occurrence of a MCY-producing cyanobacterial biomass and the aquatic macrophyte *Ceratophyllum submersum* under natural conditions). This is of particular importance since this points out a potential ecological application of the research conducted in the present project by underlining the hazardous environmental effects of a protein phosphatase inhibitor. Please see Ujvárosi et al., 2019, the list of publications prepared in the second year of project at the end of this report.

Another finding closely related to this project was that MCY-LR induced the formation of small, tonoplast coated vesicles that resembled the characteristics of autophagosomes (Nagy et al., 2018, published in the second year of project, see list of publications at the end of this report).

Task 3: Answering the question whether subcellular alterations common for ROS inducers and protein phosphatase inhibitors are due to the direct regulation of oxidative stress responses by protein phosphatases. Phosphatase inhibitor (several of them are known to induce ROS production concomitantly) treated wild-type plants will be compared to protein phosphatase mutants for completion of this task. By this approach, we will presumably detect protein phosphatase dependent cellular processes that are not related to ROS generation - therefore we will reveal whether protein phosphatase (PP1 and PP2A) inhibition and oxidative stress responses are interrelated or independent to each other.

This task was nearly completed in the second year of project and now it is fully completed. We have clearly demonstrated the validity of the main hypothesis of project: the direct relationships between the protein phosphatases PP2A (and PP1) and oxidative stress in plants. This was achieved partly by including particular phosphorylable proteins in our study (see below, achievements related to Task 3 of project).

Task 4: Detection of protein phosphatases and phosphorylable proteins involved in cellular (cytoskeletal, chromatin, organellar) oxidative stress responses. This involves a phosphoproteome approach. Cells will be treated with phosphatase inhibitors and ROS inducers/ scavengers. The next step will be the estimation of the function of these proteins.

Completion of this task is completed. Treatments of wild-type and protein phosphatase deficient model plant systems (non-treated mutants will be also used) with protein phosphatase inhibitors have been made. During our experiments, we were concentrating mainly on oxidative stress sensitive proteins directly or indirectly related to mitotic spindle organization, the cytoskeleton and chromatin

of mitotic and non-mitotic root cells, and the auxin efflux carrier proteins- the PINs. Our main findings were:

(i) We have proven that seedlings of Arabidopsis mutants in catalytic and regulatory subunits of the serine-threonine protein phosphatase PP2A are more sensitive to oxidative stress, than wild-type plants. This refers to general root development and mitotic activities of meristematic cells. Moreover, we detected alterations of chromatin structure including chromatin condensation and nuclear blebbing. Concomitantly, we observed the induction of histone H2AX hyperphosphorylation as a consequence of oxidative stress and the inhibition of serine-threonine phosphatase PP2A activity. This post-translational modification of H2AX is a known marker of DNA damage during oxidative stress. Thus, one of our main hypotheses concerning PP2A-oxidative stress relationship has been confirmed. These findings, together with the previous relevant literature, led us to publish a highly ranked review paper (Máthé et al., 2019, International Journal of Molecular Sciences, Q1). At least one more highly ranked publication related to this topic is expected relatively soon, but this needs the continuation of experimental work hopefully financed by a future project.

(ii) Studies on the protein phosphatase dependent functioning of superoxide dismutase (SOD). SOD isoforms (Mn-SOD, Cu-Zn SOD and Fe-SOD) are among the key enzymes playing important roles in the scavenging of ROS. Our project-related studies on wild-type and Arabidopsis mutants deficient in catalytic or regulatory subunits of PP2A (controls and treatments with protein phosphatase inhibitors/ROS inducers) revealed the direct relationship between the activity of SODs and the activity of serine-threonine phosphatases. This issue will be published soon. Our work will be continued in order to clarify whether PP1 or PP2A is the primary enzyme responsible for these processes (or both are equally involved) as a part of a future project.

(iii) The PIN efflux carrier proteins are playing an essential role in the transport and distribution of the plant growth regulator auxin. Thus, among many other functions they are regulating root development. Their subcellular localization depends on their reversible phosphorylation and the phosphatase playing a role in this is known to be PP2A. By the use of the phosphatase inhibitor microcystin-LR (MCY-LR) we proved that besides PP2A, PP1 is also playing a role in the regulation of PIN functioning, but this is unrelated to oxidative stress. Therefore we can state, that for distinct cellular processes, there is a relationship between PP2A(PP1) and oxidative stress, while for others there is no such relationship. However, since the organ level distribution of auxin does depend on the production of reactive oxygen species (ROS), there must be other cellular processes, partially independent to PIN, playing important roles in this. We can state that although we demonstrated the validity of our main hypothesis, the direct PP2A-ROS relationship cannot be true for all the subcellular processes. Regarding the PP2A-

PIN relationship, we have submitted a manuscript to Annals of Botany, a highly ranked journal (Freytag et al., submitted, MS no. 19812) that is now under review.

In addition to the tasks listed above, we have performed

(iv) Studies on the effects of the protein phosphatase inhibitor and ROS inducer MCY-LR on cytoskeletal and chromatin organization – mainly in mitotic cells- in the agriculturally important crops wheat and maize. This research proved that the inhibitor induces significant subcellular alterations and pave the way to practical applications of our research. These studies will contribute to a better control of cell division and growth/ improvement of crop yield for these plants.

Work fulfilled in accordance with the project:

1. Purification of MCY-LR by HPLC and CE methods. This work has been done continuously during the duration of project.
2. Treatments of wild-type and protein phosphatase deficient model plant systems with protein phosphatase inhibitors, ROS inducers and scavengers. (Immuno)histochemical and live cell imaging approaches. This work has been done and it is completed.
3. Phosphoproteome studies and the identification of proteins that link protein dephosphorylation by PP2A/PP1 to oxidative stress. Two of these protein systems-histone H2AX and SOD isoforms-were identified by molecular biology and cell biology methods. On the other hand, the functioning of PIN proteins were proven to be influenced by PP2A and PP1 without the involvement of ROS metabolism. The work is also completed.

Expected results: we will complete cytological work and gather comprehensive knowledge on the relationship between PP1/ PP2A and oxidative stress in model plants.

We now have these results in our hands completely.

The results of work for the whole duration of Project are in accordance with those expected.

Testing of main hypothesis: We will have a detailed picture on the role of different protein phosphatases on oxidative stress responses in plant cells.

According to data accumulated in the three years of project we can confirm the direct relationship between PP1/PP2A and ROS induction- the basic hypothesis of the project. On the other hand we affirm that there are also several protein phosphorylation dependent cellular processes in plants, where PP2A/PP1 mediated regulatory events cannot be related directly to oxidative stress.

Dissemination of results: we have planned 7-8 highly ranked international scientific publications and among them, probably a review article. In addition, we aimed to present our results at prestigious national and international conferences.

We have published six highly ranked publications directly related to the project, with a total impact factor of 23.46. According to the ScImago ranks, three of them are ranked Q1/D1 (Apoptosis, Frontiers in Plant Science and Science of the Total Environment), two are Q1 and one is Q2. Please note that: **(i) We have produced a higher number of top quality publications: while we produced three D1 quality papers, we originally planned two. (ii) As a result of these achievements, the PI was already invited to be a guest editor to a topical issue of Frontiers in Plant Science, a D1 journal (this topic was already launched, see URL of the Journal). The PI himself is expected to submit a paper, which can be achieved, if we find the financial resource for the OA fee ; (iii) Together with the already published papers, there is a manuscript under review and the expected number of publications proposed will be completely fulfilled. As you see in this report, other publications directly related to the project are in preparation.** In addition:

(i) we aimed a probable closing of project with a highly ranked review article on PP2A-ROS relationship in plants. This was achieved with a publication in International Journal of Molecular Sciences (Q1).

(ii) One publication, on the effects of PP2A/PP1 on PIN and auxin distribution in roots is now submitted as the seventh publication proposed. This publication (Annals of Botany, Q1) is now under review.

(iii) we have presented our results at prestigious international scientific conferences, for example the VISCEA series of Vienna Conferences on plant biology and the 11th International Botanical Microscopy Meeting of the Royal Microscopical Society in Oxford, UK: two oral and one poster presentations.

(iv) we have published a science popularization (dissemination) paper in the Hungarian popular scientific magazine Élet és Tudomány (Life and Science): “Méregkeverő cianobaktériumok- erős oxidálószerek növényi sejtekben” (Toxic cyanobacteria- strong oxidants in plant cells). We believe this outreach activity is important for making scientific research understandable and likeable for the large public.

Appendix:

Publications in the first year of project, directly related to the project (01.10.2016-30.09.2017)

1. Mathe C, M-Hamvas M, Garda T, Beyer D, Vasas G. (2017) Cellular Effects of Cylindrospermopsin (Cyanobacterial Alkaloid Toxin) and its Potential Medical Consequences. Current Medicinal Chemistry 24:91-109, Q2
2. M-Hamvas M., Ajtay K., Beyer D., Jámbrik K., Vasas G., Surányi Gy., Máthé C. (2017) Cylindrospermopsin induces biochemical changes leading to programmed cell death in plants.Apoptosis 22: 254-267, Q1/D1.

Conference participations in the first year of project, directly related to the project (01.10.2016-30.09.2017)

1. Máthé C., Mathur J., Garda T., Barton K.A.,Vámosi G. (2017) The study of plant cell dynamics with protein phosphatase inhibitors (in Hungarian) XVII. Hungarian Biologist Days, Kolozsvár, Romania, oral presentation
2. Máthé C., Garda T., Barton K.A., Wozny M., Bóka K., Böddi B., Vámosi G., Vasas G., Mathur J. (2017) The effect of microcystin-LR on division and stromule formation of Arabidopsis plastids (in Hungarian). XII. Congress of the Hungarian Society of Plant Biologists, Szeged, Hungary, oral presentation
3. Garda T., Máthé C., Vasas G., Nodzynski T. (2017) The effects of microcystin-LR ont he localization of PIN1, PIN2, PIN3 és PIN7 auxin transport proteins in *Arabidopsis thaliana* (in Hungarian). XII. Congress of the Hungarian Society of Plant Biologists, Szeged, Hungary, poster
4. M-Hamvas M., Máthé C., Jámbrik K., Ajtay K., Garda T., Surányi G., Vasas G. (2017) Changes of nuclease and protease activities related to cell death in plant test systems (in Hungarian) XII. Congress of the Hungarian Society of Plant Biologists, Szeged, Hungary, poster
5. Máthé C., Mathur J., Garda T., Barton K.A.,Vámosi G. (2017) The study of plant cell dynamics with protein phosphatase inhibitors (in Hungarian). XV. Plant Anatomy Symposium, Budapest, Hungary, oral presentation

Publications in the second year of project, directly related to the project (01.10.2017-30.09.2018)

1. Nagy M., Kéki S., Rácz D., Mathur J., Vereb G., Garda T., M-Hamvas M., Chaumont F., Bóka K., Böddi B., Freytag C., Vasas G., Máthé C. 2018. Novel fluorochromes label tonoplast in living plant cells and reveal changes in vacuolar organization after treatment with protein phosphatase inhibitors. Protoplasma 255:829-839, Q1.
2. Garda T., Kónya Z., Freytag C., Erdődi F., Gonda S., Vasas G., Szücs B., M-Hamvas M., Kiss-Szikszai A., Vámosi G., Máthé C. 2018. Allyl-isothiocyanate and microcystin-LR reveal the protein phosphatase mediated regulation of metaphase-anaphase transition in *Vicia faba.* Frontiers in Plant Science 9:1823, Q1/D1.
3. Ujvárosi A.Z., Riba M., Garda T., Gyémánt G., Vereb G., M-Hamvas M., Vasas G., Máthé C. 2019. Attack of *Microcystis aeruginosa* bloom on a *Ceratophyllum submersum* field: Ecotoxicological measurements in real environment with real microcystin exposure. Science of the Total Environment 662:735-745, Q1/ D1.

Conference participations in the second year of project, directly related to the project (01.10.2017-30.09.2018)

1. Máthé C., Garda T., Freytag Cs., Szűcs B., M-Hamvas M., Vasas G., Vámosi Gy. 2018. Allyl isothiocyanate, a horseradish metabolite, alters subcellular organization and its combination with microcystin-LR synchronizes plant cells in metaphase. XVIII. Hungarian Biologist Days, Kolozsvár, Romania (oral presentation). In addition, C. Máthé, the PI of the present project, was chair at a section of this conference.
2. Garda T., Máthé C., Papp G. V., Vasas G., Nodzynski T. 2018. Effect of microcystin-LR (MCY-LR) on auxin transport proteins in *Arabidopsis thaliana* seedlings. XVIII. Hungarian Biologist Days, Kolozsvár, Romania (oral presentation).
3. Freytag C., Szücs B., Papp G. V., Magi D., Kelemen A., Garda T., Máthé C. 2018. The effects of microcystin-LR on mitosis in different modell plants. XVIII. Hungarian Biologist Days, Kolozsvár, Romania (oral presentation).
4. M-Hamvas M., Máthé Cs., Jenei N., Ajtay K., Jámbrik K., Vasas G. 2018. The comparison of cyanotoxin induced cell death to natural senescence of cotyledons in white mustard (*Sinapis alba*)/ Cianobakteriális toxinok által indukált sejthalál folyamatok összehasonlítása a sziklevelek természetes szeneszcenciájával fehér mustár (*Sinapis alba* L.) csíranövények felhasználásával. XVIII. Hungarian Biologist Days, Kolozsvár, Romania (oral presentation).
5. Máthé C., Beyer D., Mathur J., Kónya Z., Garda T., Vasas G., M-Hamvas M., Erdődi F. 2018. Subcellular and developmental effects of serine-threonine protein phosphatase inhibition in higher plants. VISCEA- Vienna International Science Conferences and Events Association, Conference on Plant Physiology and Biochemistry, Vienna, Austria, oral presentation.

Publications in the third year of project, directly related to the project (01.10.2018-30.09.2019)

1. Máthé C, Garda T, Freytag C, M-Hamvas M (2019) The role of serine-threonine protein phosphatase PP2A in plant oxidative stress signalling-facts and hypotheses. International Journal of Molecular Sciences20: 3028. Q1
2. Freytag C, Garda T, Rigó G, Nodzyński T, Kónya Z, Erdődi F, Cséplő Á, Szabados L, Pózer E, Vasas G, Máthé C (2019) The inhibition of serine-threonine protein phosphatases PP1 and PP2A affects root development by changing the distribution of PIN proteins and auxin in Arabidopsis root tips. Annals of Botany (submitted), Q1.

Conference participations in the third year of project, directly related to the project (01.10.2018-30.09.2019)

1. Máthé C, Garda T, Freytag C, M-Hamvas M, Kelemen A, Kónya Z, Erdődi F (2019) Effects of serine-threonine protein phosphatase inhibition and ROS induction on mitotic activity, cytoskeletal and chromatin organization in model higher plants. The 11th International Botanical Microscopy Meeting of the Royal Microscopical Society in Oxford, UK. Oral presentation.

2. M-Hamvas M, Mathur J, Vereb G, Garda T, Bóka K, Böddi B, Freytag C, Vasas G, Nagy M, Kéki S, Rácz D , Chaumont F, Máthé C(2019) Novel fluorochromes (ACAIN and CACAIN) label tonoplast in living cells of Arabidopsis and *Nicotiana tabacum*. The 11th International Botanical Microscopy Meeting of the Royal Microscopical Society in Oxford, UK. Poster presentation.

References

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2. Huang, F.K.J., Naramoto, S., Zhang, J., Michniewicz, M., Offringa, R., Friml, J. (2009). PIN Auxin Efflux Carrier Polarity Is Regulated by PINOID Kinase-Mediated Recruitment into GNOM-Independent Trafficking in *Arabidopsis*. Plant Cell 21, 3839–3849. doi:10.1105/tpc.109.071639.
3. Máthé, C., Vasas, G., Borbély, G., Erdődi, F., Beyer, D., Kiss, A., Surányi, G., Gonda, S., Jámbrik, K., M-Hamvas, M. (2013): Histological, cytological and biochemical alterations induced by microcystin-LR and cylindrospermopsin in white mustard (*Sinapis alba* L.) seedlings. Acta Biologica Hungarica 64: 75-89.
4. Máthé C., Beyer D., M-Hamvas M., Vasas G. (2016) The Effects of Microcystins (cyanobacterial heptapeptides) on the Eukaryotic Cytoskeletal System. Mini-Reviews in Medicinal Chemistry, 2016, 16, 1063-1077.
5. Mathe C, M-Hamvas M, Garda T, Beyer D, Vasas G. (2017) Cellular Effects of Cylindrospermopsin (Cyanobacterial Alkaloid Toxin) and its Potential Medical Consequences. Current Medicinal Chemistry 24:91-109
6. M-Hamvas M., Ajtay K., Beyer D., Jámbrik K., Vasas G., Surányi Gy., Máthé C. (2017) Cylindrospermopsin induces biochemical changes leading to programmed cell death in plants.Apoptosis 22: 254-267.