**Final report of the 108834 K NKFIH proposal, entitled as "Examinations on the stress-protecting and physiological effects of a new, biologically active compound, S-methylmethionine salicylate"**

External application of biologically active substances can be an alternative approach to increase plants' defence potential and tolerance to various abiotic and biotic stress factors. Studies on beneficial effects of such biologically active substances, *S*-methylmethionine (SMM) and salicylic acid (SA) have provided a lot of valuable information regarding their role to counteract harmful effects of wide range of environmental and biotic stresses. To obtain a more complex and stable defence compound with an extended range of stress protecting effect, here we intended to introduce the new derivative *S*-methylmethionine salicylate (MMS), synthesised from the natural, biologically active substances SMM and SA. Since both of the original materials have a complex role as a stress-protecting compound, the new compound is expected to combine the effects of original substances and thus providing an extended stress tolerance. Here we aimed to examine and reveal the nature and mechanism of these effects on crops of outstanding agricultural importance, and compare the effect of MMS to those of the original compounds SMM and SA in order to obtain theoretical understanding and practical benefits.

**Ideal working concentration of MMS**

In order to obtain information on the effect of MMS on the basic physiological parameters of plants and determine the optimal concentration range for stress protection we examined wide range of concentrations of MMS (0.5, 0.25, 0.1 and 0.05 mM) and compared its effect to that of original natural compounds SMM, SA and the concomitant application of SMM and SA. The compounds examined were applied as a pre-treatment before setting of stress conditions at different developmental stages of plants.

Experiments with 0.5 mM concentrations of different compounds were carried out by maize hybrid Mv350. Plants were grown for 10 days in Hoagland solution of 1/4 strength and treated for 24h. Measurements were taken at the end of treatments as well as 1, 2 and 3 weeks after the treatments. Observations of these experiments: 0.5 mM MMS concentration seems to have an inhibitory effect on morphological parameters (leaf and whole plant length, fresh weight). The inhibition is most characteristic two weeks after the treatment, but the differences disappear after three weeks. Fluorescence induction parameters of chlorophyll (Chl) *a*, characteristic for functioning of electron transport chain (Fv/Fm), did not decrease significantly after the 24 h treatment (although a small Chl content decrease could be measured in all treatments). 1 and 2 weeks after the treatments the Fv/Fm values decreased in MMS-treated plants, while in case of SMM- and SA-treated plants they were similar, or slightly higher than the control values. As compared the effect of MMS to the mixture of SMM+SA, the MMS required lower concentration to influence the parameters examined, this way it proved to be more effective. After three weeks of treatments, when morphological parameters' differences disappeared between all the treated and control plants, the Fv/Fm and Chl contents were also similar.

Treatments with 0.1 mM concentrations of compounds proved to be quite similar regarding the tendencies of changes of physiological parameters, although the inhibitory effects of 0.1 mM MMS were mild as compared to 0.5 mM concentration. Using 0.05 mM concentration, no inhibitory effects of MMS could be observed.

In case of sweet corn hybrid Honey (which is planned to use in biotic stress experiments) 0.1 mM concentration was used for the preliminary examinations, since according to previous results with SMM it was more sensitive and required lower concentrations to improve defence potential against *Maize dwarf mosaic virus* (MDMV) infection. 1 week after the treatment the leaf lengths of MMS- and SMM-treated plants were smaller than the control, while the SA- and SMM+SA-treated ones were longer. 2 or 3 weeks after the treatments these differences were

equalised, except MMS-treated plants, which remain shorter. Regarding the Fv/Fm parameters there were no significant differences between the treatments after 2 weeks, but after 3 weeks a small decrease could be observed at MMS- and SMM+SA-treated plants. At the same time, according to fluorescence imaging measurements, the amount of stress-protective compounds fluorescing at 440 and 520 nm increased after all the treatments, but decreased to the level of control plants after 2 weeks of treatments, except MMS. MMS-treated plants' values were the highest during all examined period and they remained higher than the control values even after 3 weeks of treatment, indicating high amount of stress-protective materials.
According to previous results the most effective concentration of SA in abiotic stress protection proved to be 0.5 mM, and the beneficial effect of SMM could also be observed at similar and in lower concentration, either. That is why the effect of MMS on cold stress protection was investigated using both a higher (0.5 mM) and a lower (0.05 mM) concentration. The preliminary experiments also suggested that while high concentration of these compounds may reduce the germination rate and the growth of the young seedlings, low concentration may improve these parameters. According to our results on wide range of effects of MMS and comparing its effect to the mixture of SMM+SA, the MMS proved to be more effective since it required lower concentration to influence the parameters examined, this way it behaves and can be regarded as a new biologically active compound.

**SMM and MMS contribute to enhanced defence against *Maize dwarf mosaic virus* infection in sweet corn**

As compared to the information at physiological, metabolomic and gene expression levels of SA on biotic stress defence, no similar data were available on the effect of SMM. To obtain basic information before the biotic stress investigations with the combined compound MMS, preliminary experiments were carried out for the examination of SMM-caused gene expression changes during biotic stress. The effect of SMM pretreatment on the *Maize dwarf mosaic virus* (MDMV) was examined. We studied the expression changes of two genes which have specific (*GF14-6*) and general (*SAMS*) roles during viral infection. The product of *GF14-6* recognises the viral coat protein and contributes to RNA silencing, while *S*-adenosylmethionine synthase (SAMS) plays a central role in the plant sulphur metabolism and contributes to the production of several defence compounds. To study the effects of SMM, 11-d-old *Zea mays* var. *saccharata* (Sturt.) sweet corn plants were placed in Hoagland solution of ¼ strength containing 0.1 mM SMM for 24 h. MDMV infection was carried out on the 12$^{th}$ and 14$^{th}$ days of the treatment; the first and second leaves of the plants were inoculated mechanically with Dallas-A strain MDMV particles. Gene expression changes were measured 1, 2 and 3 weeks after the infections. SMM had a considerable change on the investigated genes' expression (Figure 1).

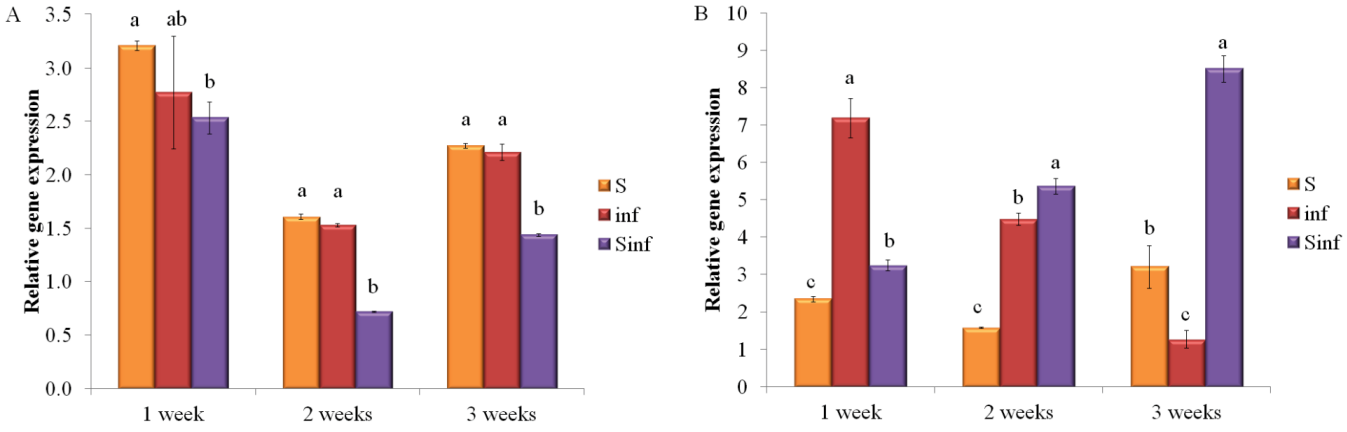


Figure 1. Changes in the relative gene expressions of *GF14-6* (A) and *SAMS* (B) of SMM-treated and MDMV-infected *Z. mays* leaves, shown in relate to control, where control equals 1 unit in all cases. Abbreviations: S - SMM-treated, inf - MDMV-infected, Sinf - SMM-pretreated followed by MDMV infection. Error bars represent standard deviation (SD), n=9. Different letters indicate significant difference ($P \leq 0.05$; DMRT).

It significantly decreased the gene expression of *GF14-6*, while infection itself resulted in a higher expression level. Since it is supposed, that the gene product of *GF14-6* regulates MDMV coat protein recognition and RNA silencing, hence the fall in the expression in SMM-pretreated and afterwards infected plants could be related to improved plant defence resulting in less virus content due to SMM. On the other hand, a more prolonged and long lasting increase is measured in *SAMS* expression as a result to SMM pretreatment followed by infection ensuring a constant long-lasting expression of this versatile enzyme involved in many aspects of gene transcription, cell housekeeping and secondary metabolite production.

Multispectral fluorescence imaging technique was applied to study changes in the concentration and distribution of Chl and defensive phenolics. MDMV infection reduced the Chl content especially in the 3$^{rd}$ week, while in SMM-pretreated plants higher Chl content was measured. SMM supported the recovery of PSII photochemical quenching and the thermal dissipation of the antennae. As typical stress syndromes, values of the combined quantum efficiency of fluorescence and constitutive thermal dissipation increased in MDMV-infected plants, especially in the 3$^{rd}$ week. When SMM was added before infection, no such increase was observed, indicating the protective effect of SMM. SMM treatment also modified the MDMV-triggered production of additional phenolics, enhancing plant defences. Results were published in Brazilian Journal of Botany 2015 38(4):771–782.

Similarly to the studies on SMM, the potential benefits of MMS pretreatment against MDMV infection in maize plants were also examined. MDMV causes breakdown of thylakoid membranes in infected mesophyll cells negatively affecting the photosynthetic electron transport and leading to formation of highly reactive oxygen species. To test the effects of MMS, we compared photosynthetic parameters characterising physiological state and levels of activity of two important antioxidant enzymes (ascorbate peroxidase (APX) and guaiacol peroxidase (GPX)). Accessing more complex evaluation of virus infection intensity, both the amount of viral coat protein (with ELISA method) and viral RNA content (with absolute quantification RT-PCR) were determined in infected plants. The results of Chl *a* fluorescence measurements showed that in MDMV-infected plants additional fluorescence quenching appear, probably as the result of associations between the virus coat protein and thylakoid membranes (Figure 2). It is important to mention that when plants were pretreated with MMS before virus infection, such associations were less formed.

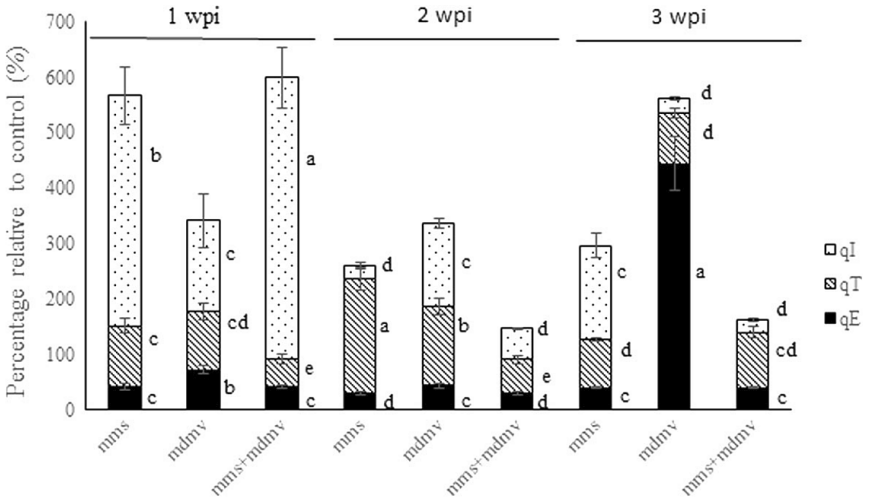



Figure 2. Changes in the NPQ parameters in MMS-treated and/or MDMV-infected maize leaves, shown in relate to control. Abbreviations: mms - MMS-treated, mdmv - MDMV-infected, mms+mdmv - MMS-pretreated followed by MDMV infection, wpi - weeks post infection. qI - photoinhibition, qT - state transition processes, qE - ΔpH-dependent quenching process of the high energy state. Total NPQ values are the sum of qI + qT + qE. Values are mean ± SD (n=6). Different letters indicate significant difference ($P \leq 0.05$; DMRT).

This statement is supported by the ELISA and absolute quantification qRT-PCR results, which showed a decrease in the amount of MDMV coat protein and viral RNA in the leaves of MMS-pretreated MDMV-infected plants and contributed to their decrease to an equal extent in maize roots, either. MDMV infection and MMS pretreatment resulted in a decrease in APX activity in maize leaves, while infection contributed to an increase in activity in the roots. Infection raised the GPX enzyme activity level, which was reduced by MMS pretreatment. These results showed that MMS pretreatment enhanced the stress response reactions against MDMV infection in maize plants and retarded the spreading of infection. Results were published in European Journal of Plant Pathology 2017 149:145–156.

**Exogenous MMS could reduce the damage of wheat plants exerted by *Blumeria graminis* f. sp. *tritici***

*Blumeria graminis* f. sp. *tritici*, which causes wheat powdery mildew, is one of the most important biotrophic wheat pathogens. The disease may cause up to 30% yield loss, thus it is important to improve the resistance of the crops against this pathogen. We aimed to examine the effects of exogenous MMS and studied whether the compound can reduce the damage exerted by *Blumeria graminis* in wheat. *Blumeria*-infected *Triticum aestivum* cv. *Carstens V* plants (Bg) were compared to infected seedlings pretreated with either 0.1 or 0.5 mM MMS (Bg+0.1M and Bg+0.5M groups), beside the untreated control (Co) and plants treated with MMS without fungal infection (0.1M and 0.5M). PSII maximum quantum efficiency was studied by fluorescence measurements, Chl content, the lengths and weights of the seedlings were also examined to study the effect of MMS.

Three days after the infection the Fv/Fm value decreased in the 2$^{nd}$ and 3$^{rd}$ leafs in Bg group. However, MMS pretreatment significantly improved the values, preventing the PSII complexes from the severe damage. Ten days after the infection the previously positive effect of the 0.1 mM MMS-pretreatment decreased, while the photosynthetic values of the 0.5 mM MMS-pretreated groups remained higher than Bg (Figure 3).

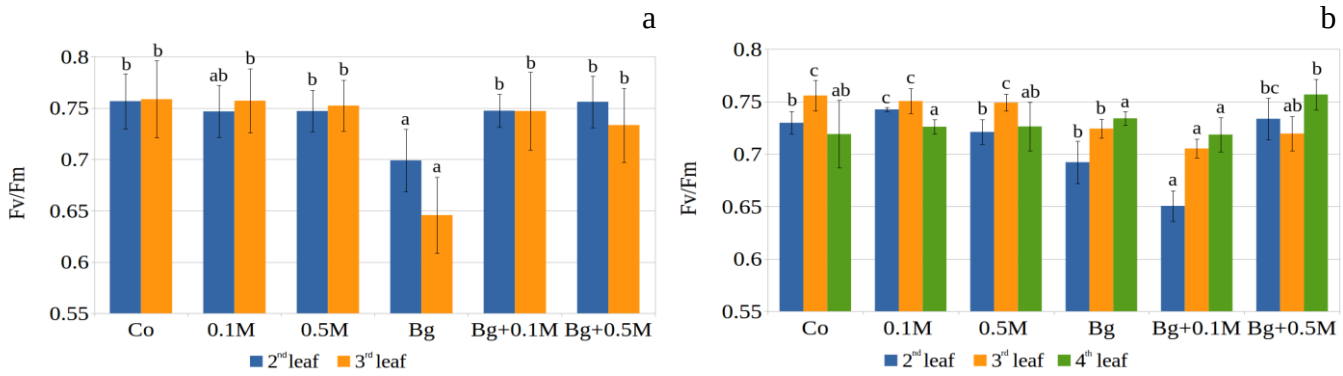



Figure 3. PSII maximum quantum efficiency (Fv/Fm, arbitrary values) measured by **a)** three days, **b)** ten days after the fungal infection. Abbreviations: Co - control, Bg - *Blumeria*-infected, 0.1M - treated or pretreated with 0.1 mM MMS, 0.5M - treated or pretreated with 0.5 mM MMS. Values are mean ± SD (n=6). Different letters indicate significant difference (P ≤ 0.05; DMRT).

Chl content changes were less unambiguous, however, the 0.5 mM pretreatment resulted in increase 3 and 10 days after the infection, as well (Figure 4). The positive effect of MMS was also manifested in the lengths and weights of the infected plants especially in the 0.5 mM MMS-pretreated ones. In accordance with our prior expectation, MMS-pretreatment preserved the function of the photosynthetic apparatus and increased the Chl content, which resulted in better physical parameters. Our results showed that exogenous MMS could reduce the damage exerted by *Blumeria graminis*. Our results were published in a poster and conference abstract (Oláh K, Balassa G, Oláh C, Rácz I, Kátay G, Vida G, Rudnóy S: The effect of S-methylmethionine-salicylate (MMS) against the biotrophic pathogen *Blumeria graminis* f. sp. *tritici*. ISBN 978-963-12-9736-2).

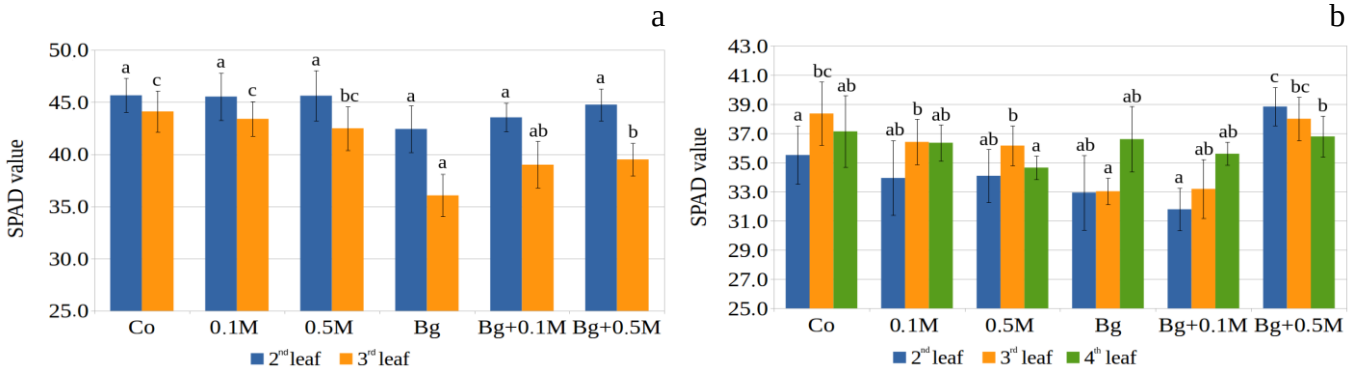



Figure 4. Chlorophyll contents (arbitrary SPAD values) of leaves measured by **a)** three days, **b)** ten days after the fungal infection. Abbreviations: Co - control, Bg - *Blumeria*-infected, 0.1M - treated or pretreated with 0.1 mM MMS, 0.5M - treated or pretreated with 0.5 mM MMS. Values are mean ± SD (n=6). Different letters indicate significant difference (P ≤ 0.05; DMRT).

**Interactions of SMM and UV-B can modify the defence mechanisms induced in maize**

Studies on the effect of exogenous SMM and UV-B radiation in young maize plants were also carried out. Seedlings of *Zea mays* L., hybrid Norma were used for the experiment. Plants were grown for 21 days with 16/8 h light/dark periods, 22/20 °C day/night temperatures, and 250 µmol $m^{-2}$ $s^{-1}$ photosynthetically active photon flux density at leaf level. Half of the plants were irradiated with excess UV light (UV dose 350 µW/$cm^2$) for 16 h during the whole light period. In the SMM treatment, 0.01 mM SMM was added to the nutrient solution from the beginning of germination to the end of the growing period.

The used moderate UV-B dose or SMM treatment did not cause substantial changes in the basic physiological parameters, however, stress-related acclimation processes could be detected in

plants treated with SMM and UV-B in combination. Increase in total phenolics and anthocyanin contents (Figure 5) and in gene expression levels of *cinnamate 4-hydroxylase* (*C4H*) and *chalcone synthase* (*CHS*) could be observed in SMM-treated plants grown at normal and excess UV light, either (Figure 6).

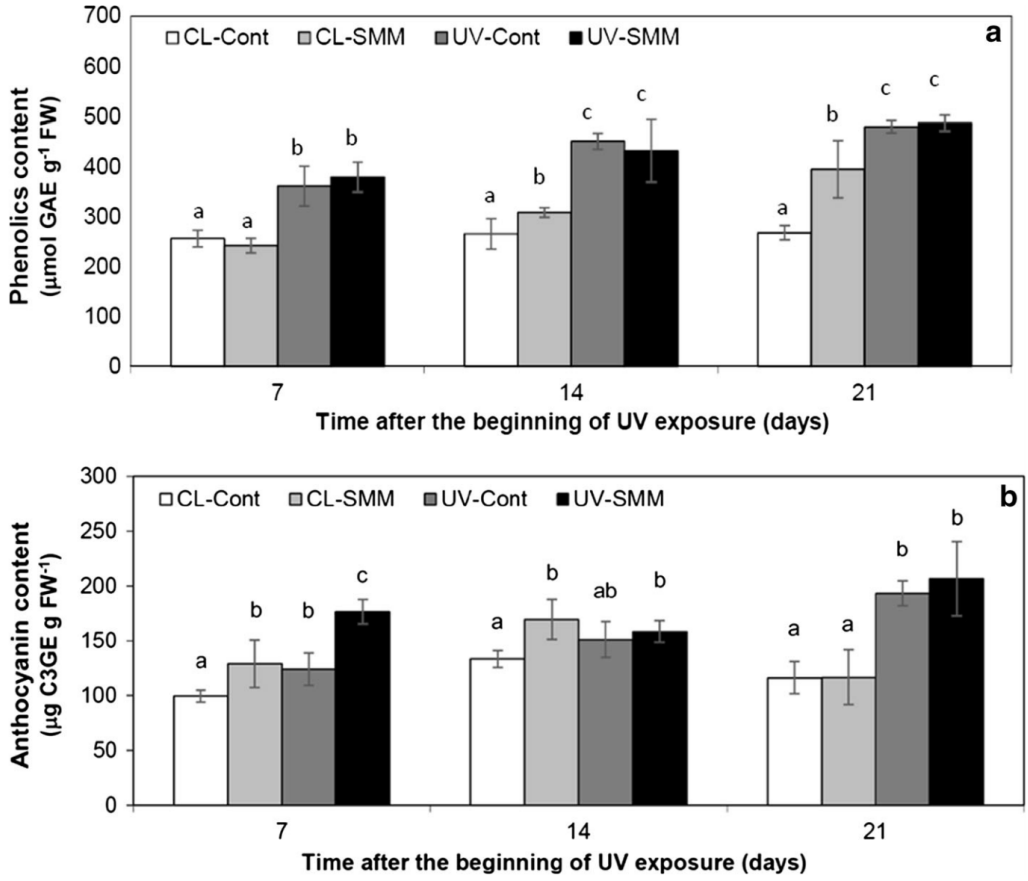
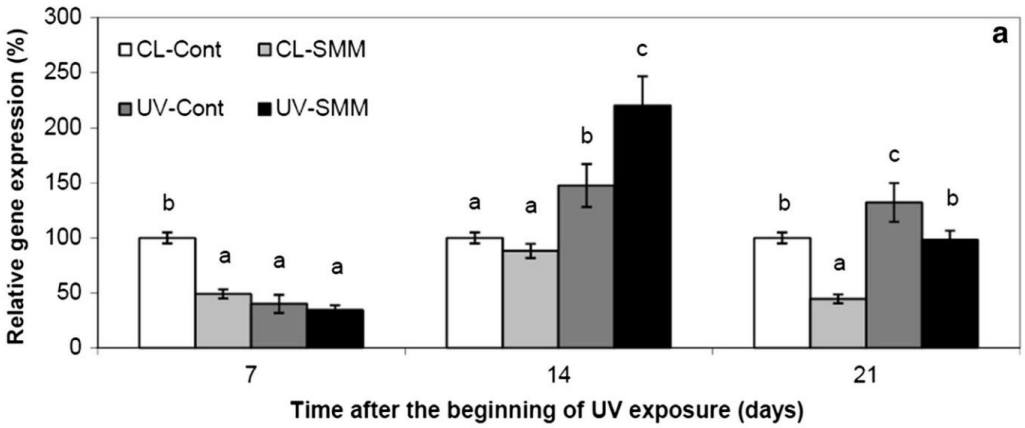



Figure 5. Effects of UV-B and SMM treatment on **a)** phenolics contents in leaves and **b)** anthocyanin contents in stems of maize seedlings. CL-Cont - control plants, CL-SMM - control light and SMM treatment, UV-Cont - UV-B exposure, UV-SMM - UV-B exposure and SMM treatment. Values are mean ± SD (n=4). Different letters indicate significant difference (P ≤ 0.05; DMRT).



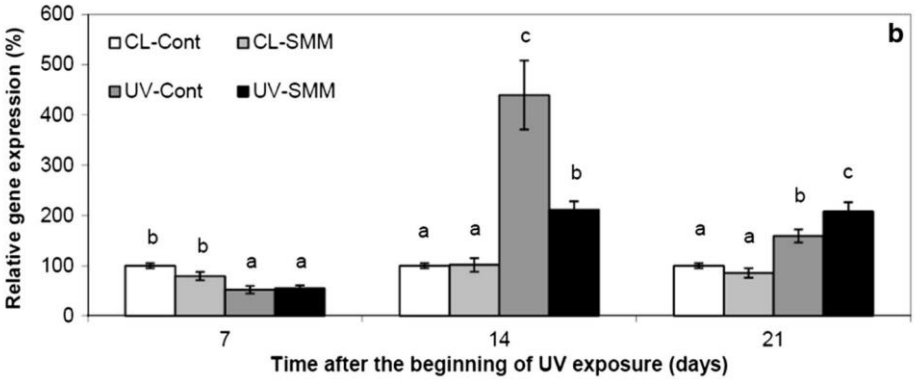



Figure 6. Relative transcription level of the phenylpropanoid biosynthetic pathway genes **a)** *C4H* and **b)** *CHS* in maize seedlings exposed to UV-B radiation and treated with SMM. Data are shown as a %, compared to control plants (100 %). CL-Cont - control plants, CL-SMM - control light and SMM treatment, UV-Cont - UV-B exposure, UV-SMM - UV-B exposure and SMM treatment. Relative gene expression was normalized by comparing the $\Delta\Delta C_t$ values of genes of interest and two internal controls. Values are mean ± SD (n=4). Different letters indicate significant difference ($P \leq 0.05$; DMRT).

In the UV-B-exposed and SMM-treated plants the upregulating effects seemed to be additive, referring to the priming character of SMM. Complex modification of activity/gene expression of certain antioxidant enzymes could also be observed. Catalase (CAT) activity showed a significant increase in UV-B- and/or SMM-treated plants, while APX only increased in SMM-treated plants. Results suggest, that both UV-B radiation and SMM treatment induce protecting mechanisms, however, the combined treatment may either accelerate or compensate each other depending on the response mechanism and the duration of the UV-B. Results were published in Acta Physiologiae Plantarum 2015 37:148 (pp 1–11).

**Enhancing the osmotic stress tolerance with the use of SMM, SA and MMS**

Investigations for beneficial effects of SMM-treatment against drought and salt stress were continued. Sweet corn (*Zea mays* L. var. *rugosa Jubilee*) seedlings were treated with 0.005 mM SMM 10 days after germination for 24 hours. During drought stress, plants were treated with 15% PEG-6000, during salt stress 60 mM NaCl was added to the growing medium, 11 days after germination. The values of stoma conductance of drought-stressed plants decreased as compared to control plants, while SMM pretreatment increased it, indicating a better water balance. It was supported by the results of gene expression changes, since SMM-pretreated drought-stressed plants had the lowest rate of gene expression of DREB2A transcription factor (located in the abscisic acid-independent pathway in an upstream position) 1 week after the treatments indicating that less aquaporin proteins needed to be synthesised (Figure 7A). Slightly different results were acquired in the case of DBP2 (transcription factor localised in the abscisic acid-dependent pathway in a more downstream position). The data indicated that this pathway is also active during osmotic stress, but lower levels of gene expression show its less importance in this process (Figure 7B). Salt stress also increased the number of reactive oxygen species in plants. During a 12-day long salt stress treatment, slight changes appeared in the gene activity of ascorbate peroxidase (APX), however, when SMM was added before salt stress, significantly higher levels of gene expression were measured, indicating that the $H_2O_2$-scavenging system was more active. The results were published in Acta Biologica Szegediensis 2014 58(1):1–5.

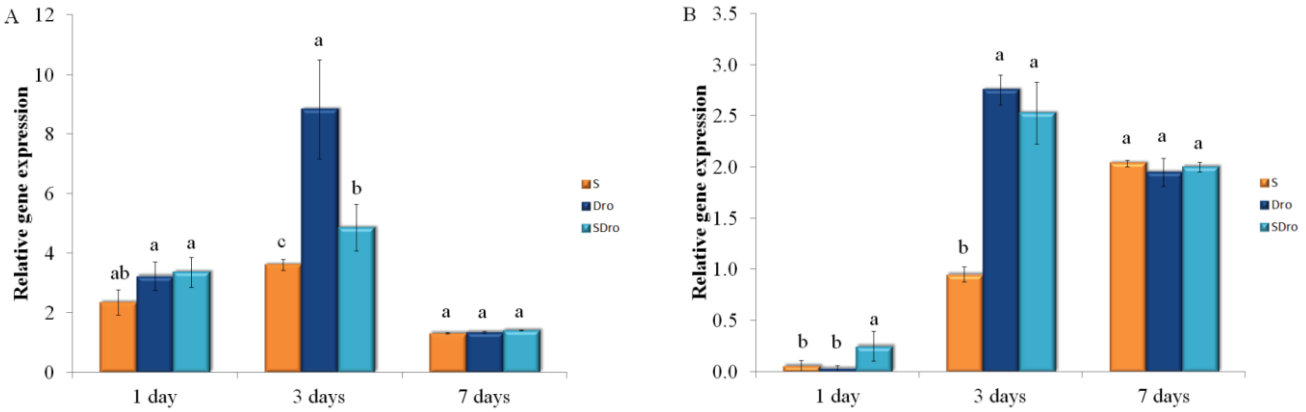



Figure 7. Changes in the relative gene expressions of *DREB2A* (A) and *DBP2* (B) of SMM-treated and drought-stressed *Z. mays* leaves, shown in relate to control, where control equals 1 unit in all cases. Abbreviations: S - SMM-treated, Dro - drought-stressed, SDro - SMM-pre-treated followed by drought stress. Values are mean ± SD (n=9). Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple-range test (DMRT).

Continuing the comparison of the protecting effect of combined new material MMS and its original compounds SMM and SA, another part of the project work was carried out to study the effects of treatment with different concentrations of SMM, MMS, sodium-salicylate (NaSA) and efficiency of different ways of treatment on some relevant metabolic activities in wheat plants under salt stress. During the work the following questions were addressed: 1. 0.5 mM SA was often used to provide protection against different stress factors in various plant species. However, under certain growth conditions this concentration may also have negative effects on plant growth and physiological parameters. Here we investigated whether a gradually increased concentration may help wheat plants to acclimate to this relatively high concentration of SA, and in parallel other protective compounds, such as SMM and MMS, and to tolerate the exposure to high salt concentration. 2. What is the effectiveness of SA, SMM and MMS; and which are the common and different mechanisms induced by these compounds? In the first experiment the investigated compounds, namely MMS, SMM and NaSA were added into the hydroponic solution of wheat plants in 2 different ways: either with a gradually increasing concentration or prompt, 2 days prior the salt treatment. In this experiment the final concentration of chemicals MMS, SMM and NaSA was 0.5 mM.

Pretreatment of wheat plants with SMM, MMS or NaSA caused a substantial decrease in the net photosynthetic rate (Pn), parallel with a decrease in the stomatal conductivity (gs). Application of a severe salt stress (200 mM NaCl) after the chemical treatment caused a substantial decrease both in Pn and gs even in the control plants (without any pretreatment). However, due to the relatively high negative values, there were no substantial differences between the treatments. In the second experiment the same treatments were repeated with a lower, 0.1 mM final concentration of MMS, SMM and NaSA, and with lower concentration of salt treatment (150 mM) 2 days after the final chemical treatments. In contrast to the first experiment, 0.1 mM concentration of the priming compounds did not reduce the Pn under control conditions. Furthermore, Pn was usually higher in the pre-treated plants than in the control ones after exposure to 150 mM NaCl (Figure 8).

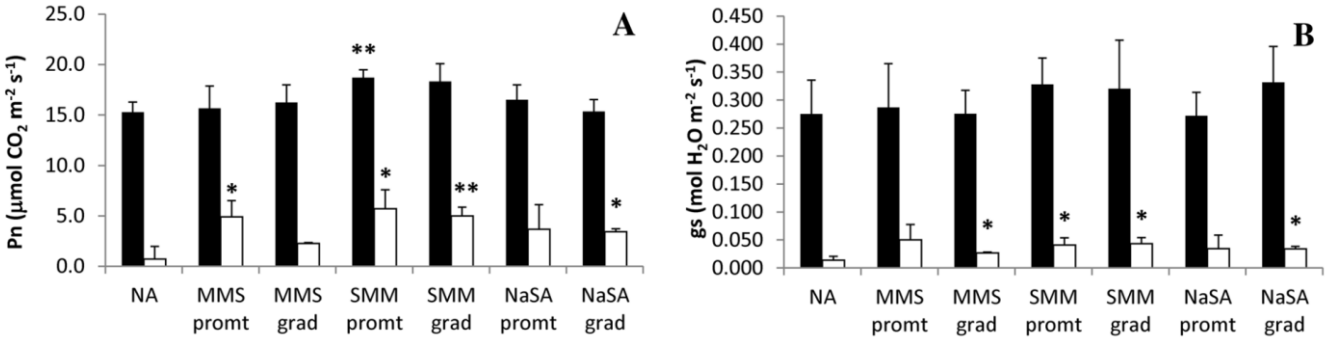



Figure 8. Effect of 150 mM NaCl treatment on gas exchange parameters, namely the net photosynthetic rate Pn (A) and stomatal conductivity gs (B) in wheat plants pretreated with 0.1 mM MMS, SMM or NaSA either in a single dose 2 days before the application of salt stress (prompt), or in a gradually increasing concentration (grad). Values are mean ± SD (n=3). * and ** represent significant differences between the non-pretreated (No addition; NA) and MMS, SMM or NaSA pretreated plants at the $P < 0.05$ and 0.01 levels, respectively.

This protection was also confirmed with chlorophyll content and fluorescence induction measurements. In order to better understand the physiological background of the protection against high salinity, certain stress-related processes have been followed. Element analyses showed that none of the protective compounds significantly altered the Na or K levels in the leaves of either the control or the salt-treated plants; so the better tolerance to high salinity is probably not due to a reduced rate of root-to-shoot $Na^+$ partitioning. However, osmotic potential measurements suggest that the physiological effects of these compounds at least partly based on the protection against the osmotic stress caused by high salinity.
The effect of MMS on the expression rates of certain genes related to salt tolerance (*NHX2*, *SOS1* and *SOS2*) was also investigated in the roots and leaves of wheat plants before and after exposure to high salinity. The biggest changes occurred in the expression rate of the *NHX2* gene in control plants 4 days after exposure to 150 mM NaCl. In plants pretreated with MMS this increase was less pronounced. Treatment with MMS alone increased the expression of *SOS1* gene in the roots, but not in the leaves. This was also observed 1 day after exposure to 150 mM NaCl in both control and MMS-treated plants. The expression rate of *SOS2* gene changed slightly in the roots and increased in the leaves of salt-treated plants after 4 days substantially. Results were published in Plant Growth Regulation 2018 85:305–315.

**MMS-pretreatment reduces the low temperature stress in maize**
Studies on improving the cold stress protection of maize using MMS-pretreatment took the largest part of the proposal work. In these experiments *Zea mays* cv. Mv350 was used. Seedlings were grown on ¼ Hoagland solution at 24 °C and 14h light period (250 µmol $m^{-2}$ $s^{-1}$ photon flux density) for 10-11 days. To determine the optimal concentration of MMS-pretreatment, plants were grown on different (0.01 – 0.1 mM) MMS containing ¼ Hoagland solution for 24 h before setting the abiotic stress conditions. Cold treatment (6 °C) was applied after MMS-treatment for 24 h (and for 4 h in some experiments). On the bases of physiological parameters (Fv/Fm, Chl content, fresh and dry weight) measured immediately or one and two weeks after cold treatment, 0.05 mM MMS proved to be optimal for pretreatment before abiotic stresses.
Low temperature decreased the actual quantum efficiency of PSII, particularly after 24-hour cold stress, which caused significantly lower values than in the control (Figure 9a). MMS-pretreated plants had higher values after 4-hour and 24-hour cold stress (ML4 and ML24), but, this difference was only significant after 24-hour chilling. In contrast, MMS treatment without cold stress (M24) did not influence the actual quantum efficiency of PSII. Non-photochemical quenching parameters showed damage to the photosynthetic apparatus, caused by low

temperature (Figure 9b). PSII electron flow (ΦPSII) significantly decreased after 4-hour and 24-hour cold stress, while the value of fluorescence and constitutive thermal dissipation (Φf,D) increased significantly in cold-stressed plants. No remarkable changes were detected in the light-dependent and ΔpH- and xanthophyll-mediated regulated thermal dissipation (ΦNPQ), but chilling induced a notable rise in the quantum efficiency of thermal dissipation by non-functional PSII reaction centres (ΦNF). MMS-pretreated plants had better values of photochemical utilization, resulting in a lower level of non-photochemical quenching.

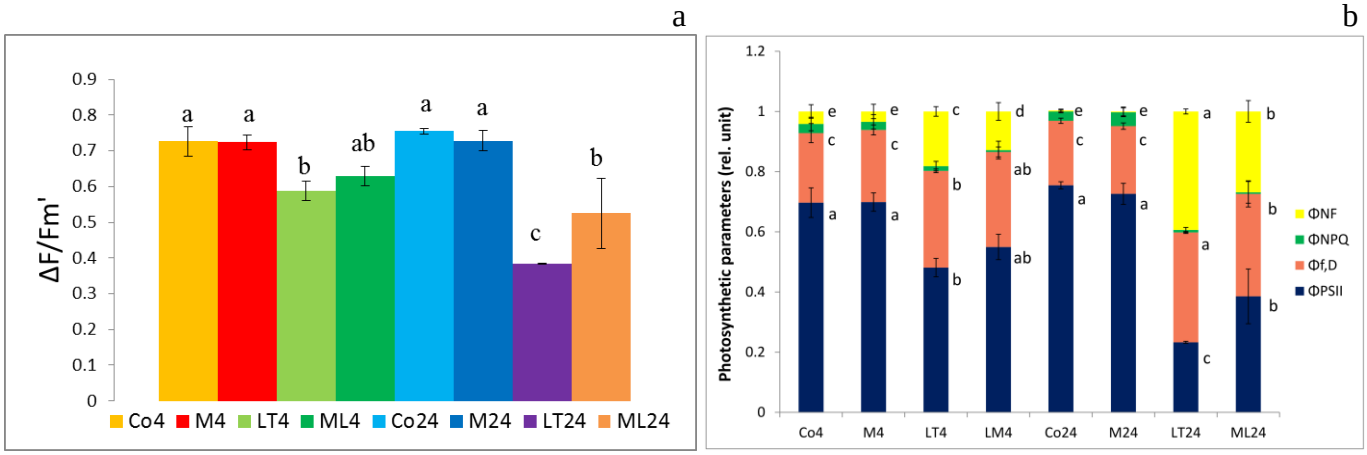



Figure 9. Photosynthetic parameters measured in cold-stressed and MMS-treated plants: **a)** actual quantum efficiency of photosystem II and **b)** changes in non-photochemical quenching parameters. Abbreviations: ΔF/Fm' - actual quantum efficiency (rel. units), ΦNF - thermal dissipation by non-functional PSII reaction centres, ΦNPQ - ΔpH- and xanthophyll-mediated regulated thermal dissipation, Φf,D - fluorescence and constitutive thermal dissipation, ΦPSII - PSII electron flow; Co4/24 - control, M4/24 - MMS-treated plants harvested 4/24 hours after MMS treatment, LT4/24 - plants exposed to 4-hour / 24-hour chilling, ML4/24 - MMS-pretreated plants exposed to 4-hour / 24-hour chilling. Values are mean ± SD (n=6). Different letters indicate significant difference (P ≤ 0.05; DMRT).

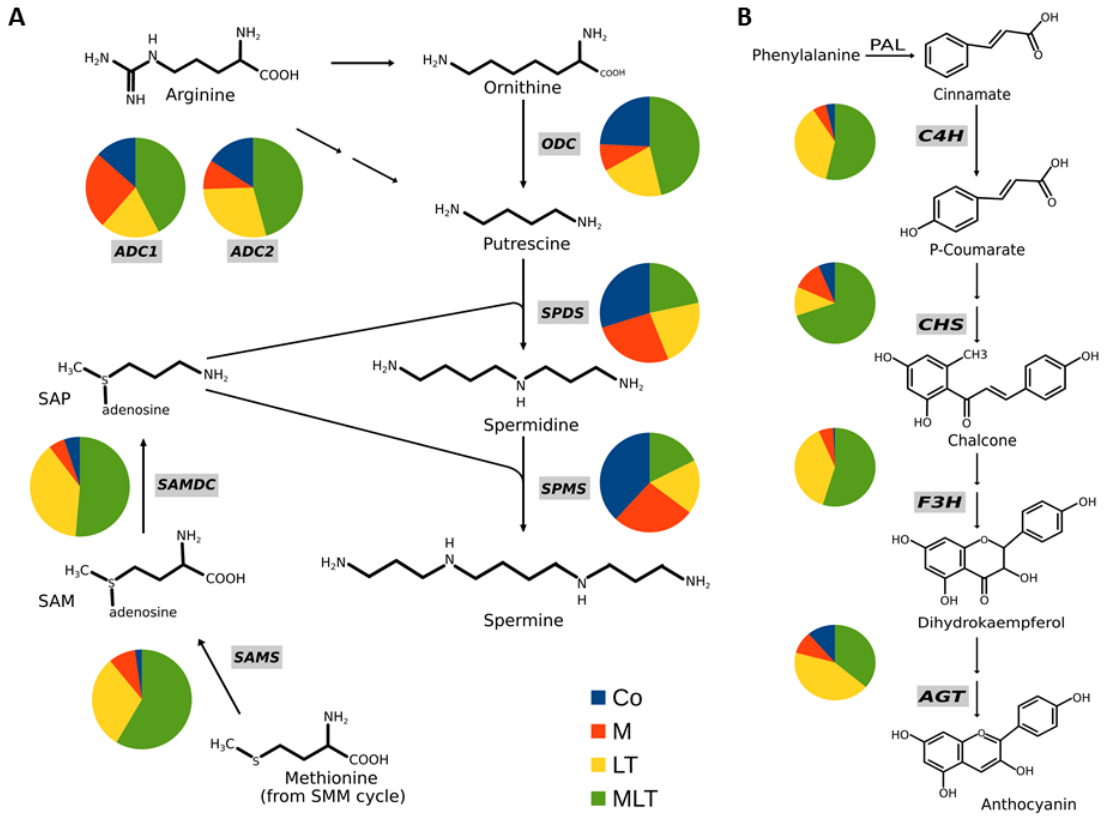


Figure 10. Relative gene expression changes in **a)** the polyamine biosynthetic pathway, and in **b)** the phenylpropanoid biosynthetic route as a result of cold stress and/or MMS-treatment. The expression of the examined genes are compared to their own controls, which values are equal to one in every pie chart. Abbreviations: Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling; SAMS - *S*-adenosylmethionine synthase; SAMDC - *S*-adenosylmethionine decarboxylase; ADC1 and ADC2 arginine decarboxylase 1 and 2; ODC - ornithine decarboxylase; SPDS - spermidine synthase; SPMS - spermine synthase; SAM - *S*-adenosylmethionine; SAP - *S*-adenosyl-methylthiopropylamine, PAL - phenylalanine ammonia-lyase, C4H - cinnamate 4-hydroxilase, CHS - chalcone synthase, F3H - flavanone 3-hydroxylase, AGT – anthocyanidin 3-O-glucosyltransferase.

Cold stress response is accompanied by massive alteration of gene expression pattern. Transcription control factors, key metabolic pathway enzymes and numerous others show significant expression changes (up- or down-regulation) during cold signalisation and acclimation. In our work we focused on two metabolic pathways having pivotal roles in abiotic and biotic stress defence: the biosynthesis and metabolism of polyamines and the phenylpropanoid biosynthetic routes up to the appearance of flavonoids (Figure 10).
To reveal changes at gene expression levels of enzymes responsible for polyamine synthesis we examined arginine-decarboxylase1 and 2 (ADC1, ADC2), ornithine decarboxylase (ODC), spermidine synthase (SPDS, coded on the 4$^{th}$ and 9$^{th}$ chromosomes), polyamine oxidase (PAO), *S*-adenosylmethionine synthase (SAMS) and *S*-adenosylmethionine decarboxylase (SAMDC), the two latter as members of SMM cycle connecting to polyamine synthesis. Highest increases were observed after combined MMS+cold treatments (Figure 10a). Even the first steps (ADC, ODC) are activated by low temperature, which is further increased by MMS-pretreatment. Elevations were characteristic after cold treatments, either, except spermidine synthase genes (including the presumptive SPMS). In the case of SAMS and SAMDC we also found significant increase in the transcription level after cold treatment, however, MMS-pretreatment more explicitly induced the gene expression. According to the expression pattern, the smallest polyamine putrescine should be the dominant cold-induced form among the metabolites in question, however, our HPLC data provide a slightly different picture, as putrescine concentration proved to be significantly higher only in the MMS-treated seedlings, compared to the control and the cold-stressed groups (Figure 11).

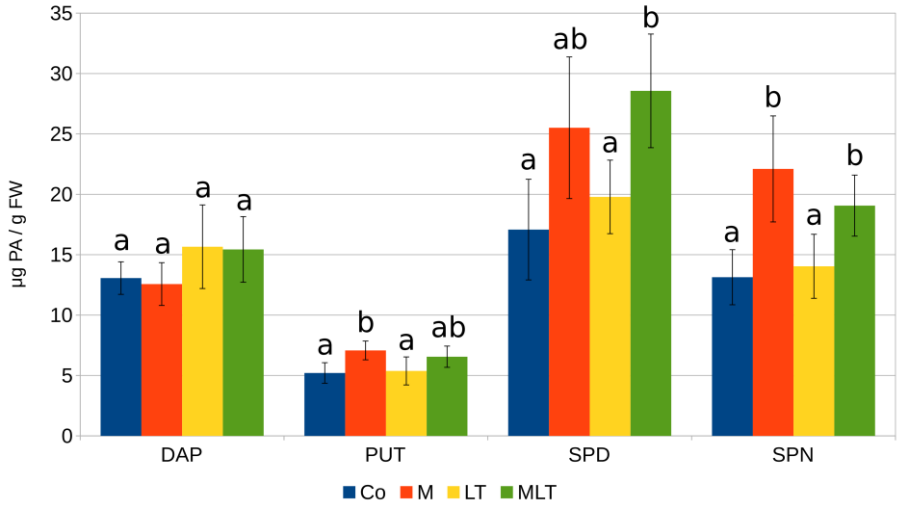



Figure 11. Changes in polyamine contents of cold-stressed and/or MMS-treated maize plants. Abbreviations: DAP - 1,3-diamino propane, PUT - putrescine, SPD – spermidine, SPN - spermine; Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling. Values are mean ± SD (n=6). Different letters indicate significant difference ($P \leq 0.05$; DMRT).

Level of spermidine was only moderately increased in reaction to the low temperature, a little more strongly after the 24-hour MMS treatment and significantly in the MMS-pretreated and cold-stressed plants, compared to the control. Spermine, the last and largest member of the homologous row of polyamines was significantly more concentrated in the two MMS-treated groups, with and without chilling. Levels of 1,3-diamino propane (DAP) did not remarkably move, showing that catabolism of polyamines was not strong in any treatment groups.
Several important metabolites synthesised in the phenylpropanoid pathway contribute to protect plants against biotic and abiotic stressors. The phenylpropanoid-based compounds include lignins, lignans, coumarins, quinones, flavonoids, anthocyanins, etc., whose roles include UV-B filtering and antioxidant properties, among others. The key enzymes in the initial steps are phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) (Figure 10b). Another important and stress-related enzyme of the pathway is chalcone synthase (CHS) using the branchpoint metabolite p-coumaroyl-CoA for the production of chalcone that is further converted to anthocyanins and other flavonoids. Flavanone 3-hydroxylase (F3H) and anthocyanidin 3-O-glucosyltransferase (AGT) two further important enzymes in the flavonoid biosynthesis branch of the pathway. MMS did not cause significant change in the expression of *C4H* and *AGT*, though a moderate increase was detected in the case of *CHS* and *F3H* (Figure 10b). Low temperature induced the pathway, which is a well-known and expected effect but MMS-pretreatment remarkably increased this induction, providing a stronger defence reaction for the pretreated plants. The total amount of phenolic compounds in the leaves did not change significantly in the various treatments compared to the control and anthocyanin content in the leaves showed a similar pattern. In the stalks, however, there was a rise in the amount of anthocyanin compounds in MMS-pretreated plants after the cold stress, though chilling did not significantly increase the anthocyanin content without MMS pretreatment. The most abundant flavonoids of maize hybrid Mv350 were isolated by HPLC method and identified by LC-MS analysis. Changes were smaller in the leaf than in the bundle sheath, where the MMS and the chilling also increased the amounts of different flavonoids (Figure 12).

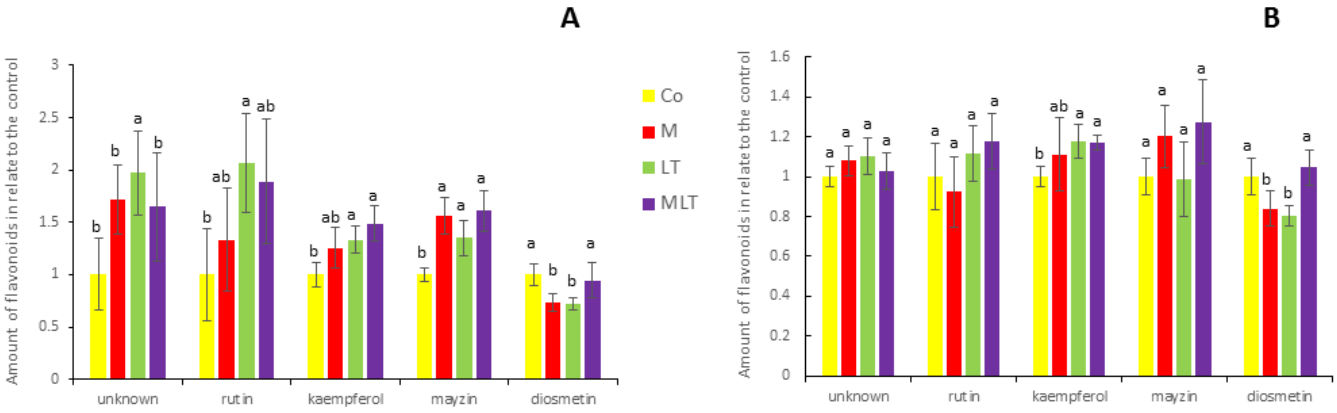



Figure 12. Changes in the contents of the dominant flavonoids of cold-stressed and/or MMS-treated maize plants, in the bundle sheath (A) and leaves (B). Abbreviations: Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling. Values are mean ± SD (n=3). Different letters indicate significant difference (P ≤ 0.05; DMRT).

Recovery from cold stress damages were also tested in maize (*Z. mays* cv. Mv350). MMS pretreatment was applied on 11 day old seedlings (0.05 mM, 24 hours), while chilling was carried out on the next day. Photosynthetic parameters (Fv/Fm and non-photochemical quenching (NPQ) data), chlorophyll content and gene expression changes were monitored on 11, 12, 13 and 14 day old maize seedlings. MMS treatment without chilling did not cause

remarkable changes in the photosynthetic parameters. When MMS was applied preliminary to the cold stress, significantly higher Fv/Fm values were measured after the chilling and partly on the first day of recovery. Also significantly better quenching parameters (higher PSII efficiency, lower energy dissipation of inactivated PSIIs, lower heat dissipation and stronger NPQ) were experienced in the MMS-pretreated plants, not only at the day of chilling but on the first day of recovery as well (Figure 13).

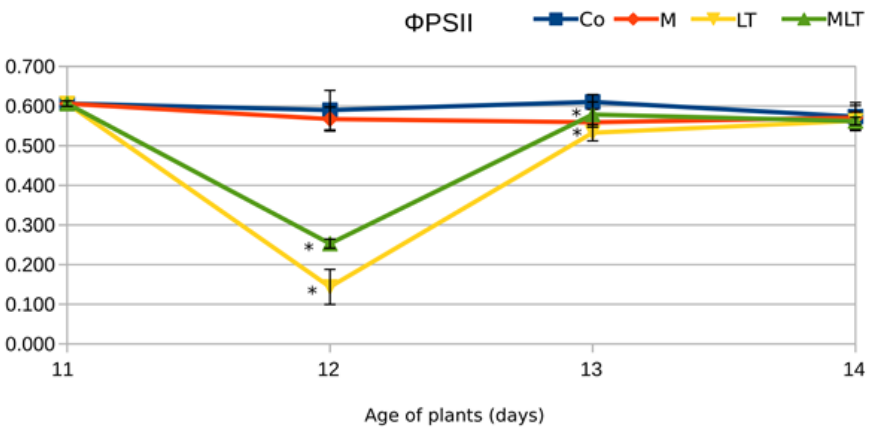



Figure 13. The efficiency of all PSII reaction centres in cold-stressed and/or MMS-treated maize plants. Abbreviations: Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling. Values are mean ± SD (n=6). Different letters indicate significant difference (P ≤ 0.05; DMRT).

Data suggest that, thanks to the beneficial effects of MMS, damages of chilling were milder in the pretreated plants and recovery started faster. Expression of certain porphyrin biosynthetic genes, being involved in the recovery of the photosynthetic apparatus, was studied by real-time qPCR. Genes were partly selected on the basis of our microarray data (see below), namely: *protochlorophyllide oxidoreductase* (*POR1*), *protoporphyrinogen IX oxidase* (*PRPO*). Besides them, two genes (*FC1*, *FC2*) of *ferrochelatase*, a key enzyme in heme biosynthetic branch of the pathway, were also studied. At last, expression changes of the NPQ pivotal element *zeaxanthin epoxidase* (*ZXE*) were measured as well. Significant differences were experienced on the 12$^{th}$ day, after the cold shock (Figure 14). *POR1* expression was intensified by either the chilling or the MMS treatment but a very impressive 25-fold increase was found only in the plants exposed to low temperature after an MMS pretreatment. Significant expression increase of *PRPO* was also experienced only in the pretreated group. *FC1* expression was induced either by cold or by MMS and also by the combination of the two treatments, while *FC2* did not show significant expression changes. *ZXE* expression was increased only by the cold stress.
In spite of the porphyrin pathway gene activations, chlorophyll data did not show significant differences in the studied period. Chlorophyll produced on the pathway could have been used to the recovery of the damaged photosynthetic systems or to the synthesis of other tetrapyrroles. Post-transcriptional inhibitory effects could also be participated.
Reviewing our results, MMS-pretreatment could help the defence mechanism of maize induced by cold stress as shown by the 12$^{th}$ day data and also could provide a faster recovery, especially shown by photosynthetic parameters.

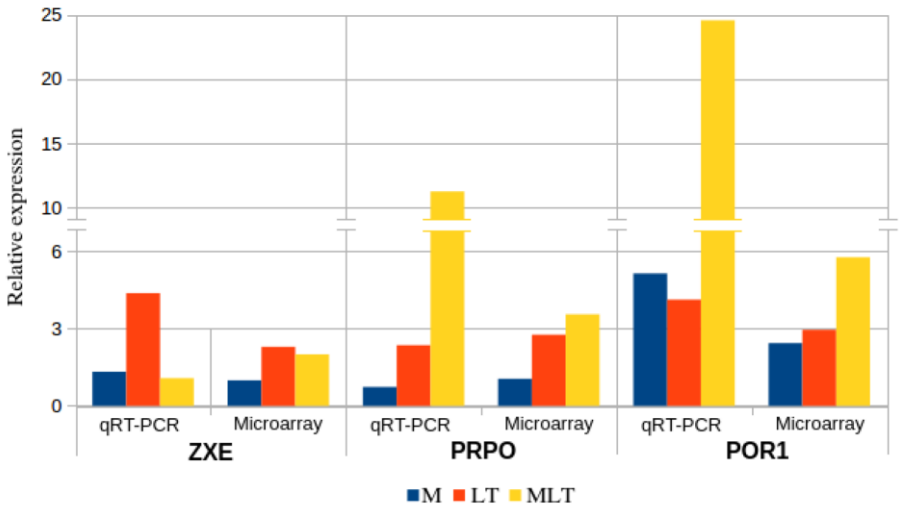



Figure 14. Comparison of the gene expression changes according to the qRT-PCR and microarray results in cold-stressed and/or MMS-treated maize plants. All data are related to the control, which equals 1. Abbreviations: ZXE - zeaxanthin epoxidase, PRPO - protoporphyrinogen IX oxidase, POR1 - protochlorophyllide oxidoreductase; Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling. Values are mean ± SD (n=6). Different letters indicate significant difference (P ≤ 0.05; DMRT).

In order to get complex information on the abiotic stress protection effect of MMS, a microarray analysis was carried out on differentially treated 14-day-old seedlings of *Z. mays* Mv350. Growing parameters and treatment methods were the same as in the abovementioned cold-stress experiments, thus the four treatment groups were the following: Co (control), M (MMS-treated without chilling), LT (low temperature-treated) and MLT (cold-stressed after the MMS pretreatment). At 5% significance level we found 15,226 gene transcripts which expression changed at least 2-fold. Changes manifested mainly in control – cold treated comparison (K-HK), but most of them exhibited expression differences in other comparisons, either. Filtering the repeated items, we could find 4,230 genes with at least 2-fold changes (decreased or increased) at least in one comparison. At 1% significance level 12,542 differentially expressed genes were found, which means changes in 3,600 genes after filtration of repeats. Gene annotation was carried out using the databases of NCBI RefSeq, SwissProt and KEGG and taking into consideration of *Z. mays*, *A. thaliana*, *O. sativa* and *T. aestivum* nucleotide and protein sequence and function data. 100% identical EST hits were found for 4,026 of the 4,230 gene probes, while 95-98% similar EST sequences for all the other genes. From these more than 4000 annotated genes of microarray (with at least 2x changes at 5% significance level) we chose 14 genes for the validation, regarding their important roles in cold stress induced signal transduction, gene expression regulation and synthesis of stress-protecting phenylpropanoids and polyamines which are highly important in cold stress response. Validation was carried out with qRT-PCR using stably expressing *PB1A10.07c membrane protein* (*MEP*), *folylpolyglutamate synthase* (*FPGS*) and *Leunig* (*LUG*) as internal reference genes. Examining gene expression levels of pivotal transcription factors the membrane permeability responsible *LOX* (*lipoxygenase*) was highest at combined, cold response regulating *ICE* (*Inducer of CBF Expression*) in cold and combined, while *DREB* (*dehydration responsive element binding*) in cold treatment. Regarding the changes in gene expression levels of phenylpropanoid pathway enzymes (flavanone 3-hydroxylase, cinnamic acid 4-hydroxylase, chalcone synthase and anthocyanidin 3-O-glucosyltransferase) all were highest in MMS-pretreated and cold-stressed plants, while isoflavone 2'-hydroxylase expression in cold-treated plants. The MMS treatment alone not resulted significant changes as compared to control plants. In case of other metabolically important enzyme coding genes (*serine acetyltransferase*, *protoporphyrinogen*

*IX oxidase* and *zeaxanthin epoxidase*) the MMS treatment caused low level of expression increase in addition to the highest increase during the combined treatment. Trends of qRT-PCR measurements were similar to microarray, however, due to higher sensitivity, expression levels were higher (Figure 14).

Linear regression analysis showed significant correlation between the microarray and real-time PCR data (R=0.8421). In order to the better perception of the effects of MMS pretreatment on chilling-stressed plants, we carried out a principal component analysis (PCA) on the $log_2$FC values of the microarray analysis. According to the PCA results we could identify two from the six principal components, covering 93% of the variance, thus explaining the majority of the data. The first principal component (PC1) includes 66.64% of the data and has a moderate negative correlation with $log_2$FC values of LT-M, Co-LT, MLT-M and MLT-Co comparisons. This assumes the significant influence of chilling treatment on the gene expression pattern, however, the effect of combined treatment also appears. The second principal component (PC2) includes 27.14% of the data and strongly correlates with the $log_2$FC values of LT-MLT and Co-M comparisons, which is supposed to be the effect of combined treatment on the gene expression pattern. According to the results PC1 and PC2 refer to chilling and MMS effect, respectively, which may help to clarify the genes of interest for further examinations. (Figure 15).

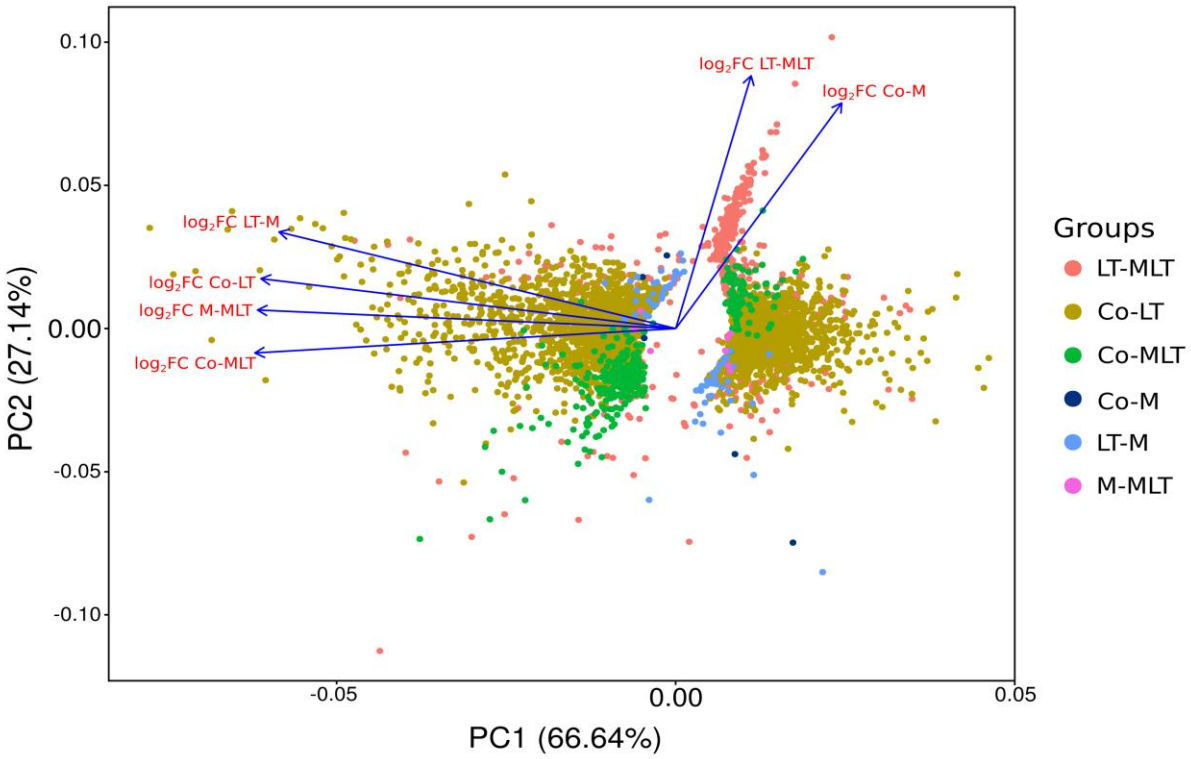



Figure 15. A scatterplot diagram of the expression datasets derived from principal component analysis based on the $log_2$FC values of the genes that provided significant changes according to the result of the microarray dataset. All genes were plotted with respect to the first and second principal components (PC1 and PC2). Abbreviations: Co - control, M - MMS-treated plants harvested 24 hours after MMS treatment, LT - plants exposed to 24-hour chilling, MLT - MMS-pretreated plants exposed to 24-hour chilling.

Gene functions were analysed by GO annotation, which by the following three main categories are determined: *biological process*, *molecular function* or *cellular component*. The *biological process* analysis is presented using the ClueGO software (Figure 16). The graphic illustrates the major changes occurred in each treatment. We could identify five hits (photosynthesis,

cellular amino acid, sulphur compound, porphyrin and ROS metabolic processes), that not only belong to the chilling stress response but seem to be the effect of MMS-pretreatment on the gene expression pattern. These GO terms represent metabolic processes where SMM, one of the components of MMS, exposes its effect resulting in gene expression changes. Furthermore, we could identify numerous other GO groups that form a complex network and reveal a highly integrated regulatory system. These consist of such as response to hormone stimulus, ROS and oxidative stress, which regulates cellular and metabolic processes, thus stress response. This result provides more detailed information and is in accordance with the PCA result, thus also indicated the effect of MMS.

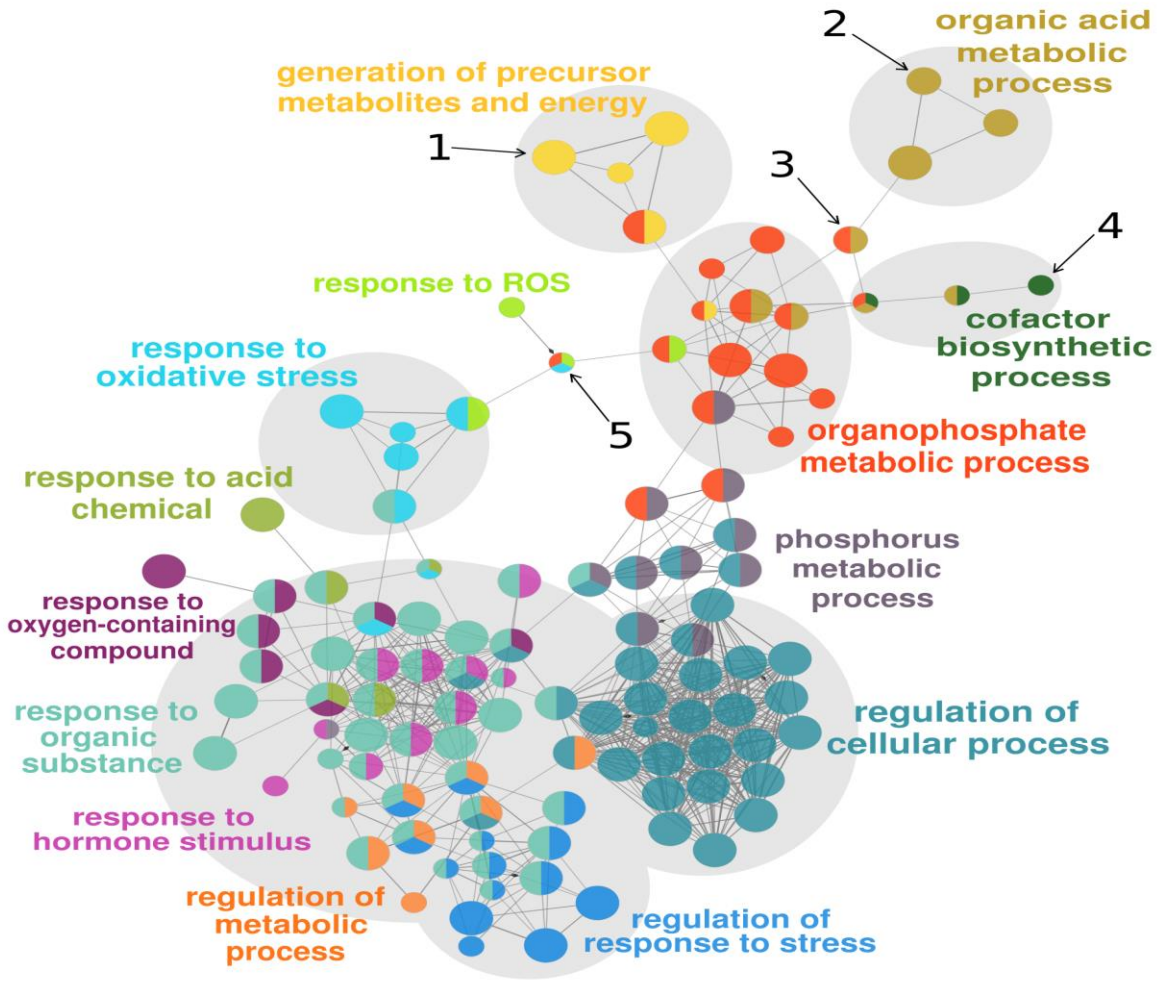



Figure 16. Result of the ClueGO analysis, showing the most affected *biological process* categories and their connections. Size of the circles is directly proportional to the level of significance. The graphic was created with a $\kappa$ – score 3 and contains the significant matches ($P \leq 0.05$) of GO groups found by the software. Numbers refer to: 1 – photosynthesis; 2 – cellular amino acid metabolic process; 3 – sulphur compound metabolic process; 4 – porphyrin and chlorophyll metabolism; 5 – ROS metabolic process.

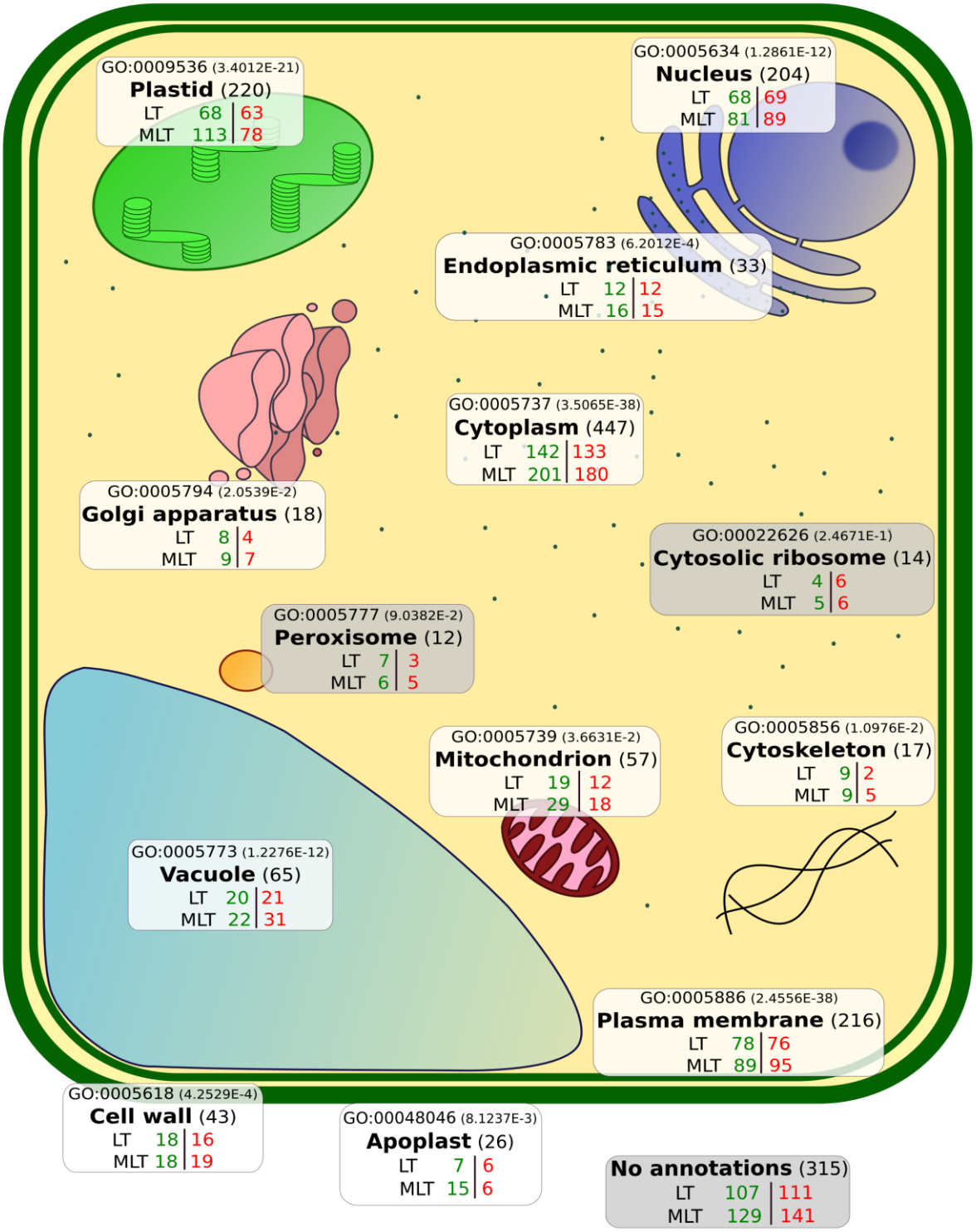



Figure 17. Result of the GO analysis representing the number of *cellular components*-related genes that showed significant expression change. The number in parenthesis found after the GO group's name indicates the maximum number of such genes found in any treatment comparison. Green and red numbers indicate the number of up- and down-regulated *cellular components*-related genes, respectively, with significantly changing expression data in the LT and MLT groups, related to the control. The significant matches ($P \leq 0.05$) are in white boxes. Grey boxes represent the non-significant matches and number of genes with unknown localisation.

The *molecular function* GO category did not show remarkable changes, on the contrary, numerous genes classified into the *cellular component* category proved to be up- and

downregulated in reaction to the applied treatments (Figure 17). *Cellular component*-related gene expression changes were the most considerable after the combined treatment (MLT) compared to the cold-treated or control plants. Remarkable differences were observed in the number of up- and downregulated genes connected to the chloroplast, cytoplasm, nucleus or plasma membrane. We found the most numerous changes in connection to the cytoplasm: expression of 447 cytoplasm-related genes showed at least two-fold change, either up- or downregulated. The changes was more pronounced in the MLT group. Among the affected cytoplasm-related genes there were those coding for transcription factors, ribosomal proteins, auxin- and other hormone-related factors, AAA+-type ATPases, 14-3-3-like proteins, hydroxyproline-rich glycoproteins, photoreceptor-related proteins, pumps and channels and last but not least proteasome-related factors, showing that gene activation could mean protein degradation as well. In the case of the chloroplast also numerous genes proved to be controlled, in MMS-pretreated and cold-stressed plants (MLT) more than 100 genes were activated, coding for members of the photosynthetic apparatus and ATP-synthesising complex, nucleic acid metabolism-related proteins, members of the Calvin cycle, chlorophyll-binding proteins, ABC-transporters and the plastidic isoleucine-tRNA ligase. Genes related to the plasma membrane were also represented in a relatively high number among the genes with significantly changed expression level (216 genes, including those for aquaporins and many further transporters, receptor-like and other kinases, actin, myosin, antioxidant enzymes, phototropin and numerous factors related to signal transduction). The nucleus was proved to be the fourth most affected compartment with 204 genes, for instance members of the nucleic acid metabolism (nucleotide kinases, helicases, PCNA…), transcription factors (G-box bZIP factors, bHLH Scream2, DREB1D, auxin-related factors, WRKY33…), chromatin modifiers (histone deacetylase HDT2, lysine methyltransferase), cyclin-B1, CDK-inhibitor 5, and countless members of the fine-tuning of gene expression (RNA-dependent RNA polymerase 2, elongation factor 2, eukaryotic translation initiation factor isoform 4G-1, polyadenylation and cleavage factor homolog 4, SUMO1 etc.).
Our results have partially been published in Russian Journal of Plant Physiology 2017 65(1):63–68, while the larger part of the data, including the microarray analysis, is proposed to publish in a D1 plant science journal this summer.

**General consequences**

As represented in the Figure 17, a general consequence was that the cold stress with and without the preliminary MMS-treatment resulted in higher number of upregulation than repression in the gene regulation pattern. Our microarray and qRT-PCR gene expression data coincide with the physiological and naked-eye observations showing that MMS caused a moderate stress that could stimulate the intrinsic defence system of maize, making that capable of coping with a subsequent stronger stress effect more easily, faster and effectively, resulting in less severe damages and faster regeneration. As summarising our results, we can conclude that this priming effect appears in all experiments without regards of the nature of the applied stress type: abiotic and biotic stress factors, that can more or less severely damage plant tissues, caused significantly lesser problems when a preliminary MMS-treatment helped the plant preparing for the given shock or stress. Plant's physiological activity was increased, the general and special defence systems were activated and health of the challenged plants were less damaged. Growing parameter measurements showed that growth was relatively unaffected by MMS, thus the more effective defence did not result in growth decrease. This could be explained by the fact that priming is not really costly, as it rather makes defence faster but not more resource-consuming: prevention of the early damages in cell structures and expensive molecules could make plant defence more effective and cheaper.

Practical application of MMS is hopeful. The two most reliable ways are 1) treatments of seeds before sowing (seed soaking) and/or 2) spraying of young or adult plants. Seed treatment could support germination and help seedlings to cope with the first environmental challenges in the field but the effect would give out as the plants grow. Spraying could be applied in any phenophase, however, it is not easy to exploit its maximal efficiency, since it is the best as a pretreatment agent, preliminary to a severe stress effect. We presume that either the exclusive usage of one of the two methods or the combination of them could help the culture of many different crop species, after an indispensable optimisation procedure. Based on our laboratory experience, preliminary arable experiments had begun, however, this is a long-term process, which results could be analysed years hence.