Physiological and Molecular Background of Maize Cold-Tolerance Enhancement with S-methylmethionine Salicylate

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Abstract

Low temperature is amongst the most influential abiotic stress factors, having deep impact on plant growth, yield and productivity. Studies on beneficial effects of certain 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 environmental stresses such as chilling. To obtain a more complex and stable defence compound with an extended range of stress-protective effect, the new derivative S-methylmethionine salicylate (MMS) was synthesised from the natural, biologically active substances SMM and SA. Since both original materials have complex stress-protective roles, the new compound was expected to combine the effects of original substances and to stabilise the unstable SMM in the new compound, thus providing an extended stress tolerance. Photosynthetic efficiency and accumulation of stress-related metabolites (polyamines and flavonoids) were measured in chilled and control plants, with and without MMS pretreatment, and expression changes of several genes involved in the cold stress response were analysed by quantitative real-time PCR (RT-qPCR) and a detailed microarray study. Our data show how the MMS combines the effect of SMM and SA on molecular level, causing numerous changes in the gene expression pattern and metabolite content. MMS gives rise to a better physiological condition, thus it could provide an alternative, environmental friendly way to enhance the plants defence mechanisms against stressors. As MMS is more stable than SMM, it promises easier, more long-lasting and more cost-effective usage in agriculture, with a complementing effect of SA.

Keywords Maize · Cold stress · Priming · Transcriptomics · Metabolomics

Introduction

Low temperature is amongst the most influential abiotic stress factors, having deep impact on plant growth, yield and productivity. Crops of tropical or subtropical origin like maize are especially sensitive to chilling: temperatures below 10 °C can severely damage their structure and metabolic processes. Deteriorated enzyme activities, decreased activity of photosynthesis, appearance of reactive oxygen species (ROS) and protein denaturation are amongst the most known effects of cold stress. Chilled plants sense temperature decrease at different sensory levels including the perception of membrane fluidity changes and of the altered structure and function of individual and multimeric proteins (Mehrotra et al. 2020). Perception of chilling triggers a typical cold stress response pathway, with prominent roles of the Ca2+-dependent and mitogen-activated protein kinase cascades (CDPK and MAPK, respectively), phospholipases, ICE-CBF/DREB and further transcription factors in signal
transduction and activated cold-responsive (COR) genes in physiological responses to cold tolerance (Ding et al. 2019; Mehrrotra et al. 2020). C-repeat/DREB binding factors (CBFs) proved to be key transcription factors in cold stress answers and cold acclimation. At the level of transcription factors, cold stress interacts to the circadian rhythm, the hormonal homeostasis and the light signalling, amongst others (Shi et al. 2018).

The positively charged aliphatic amine polyamines also have essential roles in cold acclimation and other abiotic and biotic stress responses, though they are involved in the usual plant development as well (Chen et al. 2019; Pál et al. 2015). Ethylene-responsive factors, important components in cold stress regulation, were also showed to enhance the turnover of polyamines (Zhou et al. 2018). Putrescine, spermidine and spermine the most common polyamines all were assumed to directly protect the membranes and macromolecules; however, it is more and more obvious that they are much more important as signalling molecules and elicitors of secondary metabolites (Pál et al. 2015; Mustafavi et al. 2018). Phenolics are plant secondary metabolites and are also amongst the most significant stress-protective compounds of all plants. Their biosynthesis is connected to the shikimate-phenylpropanoid pathway, where the initial steps are catalysed sequentially by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and coumaroyl-CoA ligase (4CL), resulting in coumaroyl-CoA, a key compound of the pathway (Vogt 2010; Shuab et al. 2016). Biosynthesis of many further phenolics is branched from this point, including that of flavonoids, the most known representatives of phenolics, which synthesis starts with the action of chalcone synthase (CHS). It has been proved that cold stress significantly increases the activity of the phenylpropanoid biosynthetic pathway, resulting in numerous defence compounds (Li et al. 2019).

Improving the stress tolerance of crops is a principal task in agriculture. Besides the time-consuming traditional and the expensive transgenic ways of breeding, 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 stresses such as chilling (Rácz et al. 2008; Janda et al. 2017). SMM, the most important transported sulphur metabolite of plants, has been proved to provide significant protection to maize plants against different stress factors, especially low temperature (Páldi et al. 2014; Rudnóy et al. 2015). SA, this phenolic-type plant hormone, was primarily shown as a key player in biotic stress responses, though its role in the defence against chilling and other abiotic stress situations also evidenced later (Miusa et al. 2013; Janda et al. 2017). To obtain a more complex and stable defence compound with an extended range of stress-protective effect, the new derivative S-methylmethionine salicylate (MMS) was synthesised from the natural, biologically active substances SMM and SA. Since both original materials have complex stress-protective roles, the new compound was expected to combine the effects of original substances and to stabilise the unstable SMM in the new compound, thus providing an extended stress tolerance (Janda et al. 2018; Oláh et al. 2018). Here, we aimed to examine and reveal the nature and mechanism of these effects on maize and compare the effect of MMS to those of the original compounds SMM and SA in order to acquire theoretical understanding and practical benefits. Photosynthetic efficiency and accumulation of stress-related metabolites (polyamines and flavonoids) were measured in chilled and control plants, with and without MMS pretreatment, and expression changes of several genes involved in the polyamine or flavonoid biosynthesis or cold stress response were analysed by quantitative real-time PCR (RT-qPCR) and a detailed microarray study.

Materials and Methods

Plant Material, Growth Conditions and Treatments

Plants (Zea mays L. cv. Mv350) were grown in hydroponics in a climate chamber (14-h photoperiod, photosynthetic photon flux density (PPFD) of 200 μmol m⁻² s⁻¹, 23 °C and ~75% relative humidity). 3-day-old germinated kernels were transferred to a stainless-steel grid in 800-ml Erlenmeyer flasks containing 600 ml ¼ strength Hoagland solution, that was changed every second day. Since MMS is a derivative of SMM and SA and likely it dissociates in aqueous solutions, its effective concentration was chosen based on the experience gained with the two precursors (Rácz et al. 2008; Janda et al. 2017). After preliminary experiments, 0.05 mM has been proven to the most effective and not harmful concentration (Oláh et al. 2018). 24-h MMS treatment was carried out on 10-day-old plants, by adding water-dissolved MMS to the hydroponic solution in a final concentration of 0.05 mM, while cold stress was applied on 11-day-old plants by changing their room temperature growing solution to 6 °C growing solution and transferring them to a 6 °C growth room for 24 h. PPFD was reduced to 130 μmol m⁻² s⁻¹ in the cold chamber in order to avoid photoinhibition. Our sample plants were separated into 4 groups: Co—non-stressed control plants; M—MMS-treated plants; LT—plants exposed to low temperature; MLT: MMS-pretreated plants exposed to low temperature. All measurements were carried out on 12-day-old plants, thus immediately after the cold treatment (LT, MLT) or at the
same time in the case of plants not exposed to low temperature (Co or M).

**Electrolyte Leakage Analysis**

Electrolyte leakage (EL) was measured according to Campos et al. (2003), with some modifications. Three leaf discs (d = 1 cm) per sample were used from each treatment groups, right after the 24-h cold treatment and at the same time for the groups without cold stress. In order to remove the surface-adhered electrolytes, the samples were washed with de-ionised water and then incubated in 10 ml of de-ionised water on a rotary shaker (100 rpm, 25 °C). The electrical conductivity of the bathing solution was measured after 1, 2, 3 and 4 h during the incubation, using a Crison EC-Meter Basic 30+(Crison Instruments, Spain). The electrolyte leakage % was expressed as the percentage of the maximum conductivity of the sample that was measured after freezing and boiling the samples (Campos et al. 2003).

**Antioxidant Enzyme Activity Measurements**

Superoxide dismutase (SOD) enzyme activity was measured according to the method elaborated by Smette et al. (2005) as described in Solti et al. (2016). The activity of SOD isoenzymes was defined by native acrylamide gel separation and negative activity staining (15 min preincubation in dark and 30 min of illumination). The densitometry and evaluation of gels was conducted using Phoretix 4.01 software (Phoretix International, Newcastle upon Tyne, UK). The activity of ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes was measured by spectrophotometric method (Janda et al. 1999). APX and GR enzyme reactions were detected at 290 nm and 412 nm, respectively. The results were normalised according to the protein content of the samples (ΔA min⁻¹ g⁻¹ protein). The total protein content in enzyme extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

**Chlorophyll Fluorescence Measurements and Chlorophyll Content Determination**

Chlorophyll a fluorescence was analysed by a PAM-101-102-103 fluorometer (Walz, Effeltrich, Germany). Second leaves were measured after 20 min of dark adaptation. The maximum quantum efficiency of photosystem II (PSII) (Fv/Fm), calculated from the values of minimum (F0) and maximum fluorescence (Fm), was determined as (Fv/Fm = (Fm − F0)/Fm). F0 was determined by switching on the measuring light with modulation frequency of 1.6 kHz and PPFD less than 1 µmol m⁻² s⁻¹ after 3 s illumination by far-red light in order to eliminate reduced electron carriers. Fm and Fm' values were measured using a 0.7 s flash of white light at PPFD 3500 µmol m⁻² s⁻¹ (light source: KL 1500 electronic, Schott, Mainz, Germany; Solti et al. 2008). For quenching analysis, the leaves were exposed to actinic white light (PPFD of 100 µmol photons m⁻² s⁻¹) for 20 min. Excitation energy allocations were calculated according to Hendrickson et al. (2005), where parameters are as follows: Φ_LD—fluorescence and constitutive thermal dissipation; Φ_NF—thermal dissipation in non-functional PSII; Φ_NPQ—light-dependent and ΔpH- and xanthophyll-mediated regulated thermal dissipation; Φ_PSI—quantum yield of PSII electron flow. The distribution of the parameters were calculated according to the following equation: 1 = Φ_PSI + Φ_LD + Φ_NPQ + Φ_NF, F_vM/F_mM was applied as the mean of F_v/F_m values of Co (quasi non-inhibited) plants, according to Solti et al. (2014).

Chlorophylls were extracted from the 2nd leaves with 80% (v/v) acetone. The chlorophyll content was determined spectrophotometrically using the extinction coefficients of Porra et al. (1989).

**Proteomic Analysis of the Photosynthetic Apparatus**

Isolation of mesophyll thylakoids was carried out from the second leaves of the differently treated maize seedlings (two biological replicates). The middle parts of mature leaves were homogenized under 4 °C in isolation buffer (50 mM HEPES–KOH, pH 7.0, 330 mM sorbitol, 2 mM EDTA, 2 mM MgCl₂, 0.1% (w/V) BSA, 0.1% (w/V) Na-ascorbate) by Waring Blendor (Waring Commercial, Stamford, CT, USA) for 2 × 3 s. Homogenate was filtered on 4 layers of gauze and 2 layers of Miracloth (Merck KGaA, Darmstadt, Germany), and centrifuged at 1500 g for 2 min. The pellet was washed in the homogenisation buffer (2000 g for 5 min), osmotically broken in 10 mM Na₄P₂O₇ (pH 7.4), 5 mM MgCl₂, and centrifuged (5000 g for 5 min). To remove most CF₁, pelleted thylakoids were washed 5 mM Tricine-(CH₃)₄NOH (pH 7.5), 0.1 M sorbitol, 10,000 g for 10 min. At last, the pellet was stored in 2 mM Tris-maleate (pH 7.0), 40% (V/V) glycerol in liquid nitrogen.

Before applying to Blue-Native gels to separate thylakoid complexes, thylakoids were washed in 50 mM Bis-Tris-HCl (pH 7.0) containing 330 mM sorbitol and 250 µg mL⁻¹ Pefabloc, and solubilized (0.5 mg Chl mL⁻¹ in all treatments) in 50 mM Bis-Tris-HCl (pH 7.0) containing 750 mM e-aminoacaproic acid, 0.5 mM EDTA, 250 µg mL⁻¹ Pefabloc with a mixture of 1% (w/V) n-dodecyl-β-d-maltoside and 1% (w/V) digitonin (SERVA) on ice for 30 min. After a 15-min centrifugation at 18000 g and 4 °C, the supernatant was supplemented with 1/5 volume of 5% (w/V) Serva Blue G dissolved in 500 mM e-aminoacaproic acid. 10 or 15 µL of the solubilised material was applied per lanes. Total density of lanes was directly proportional to the applied amount of Chl (thylakoids) up to 20 µL of samples. To separate thylakoid
complexes, first-dimension electrophoresis was performed under native conditions by BN-PAGE (Kügler et al. 1997) using 4.5–12% w/v acrylamide gradient gels (Mini-Protean, BioRad). Electrophoresis was carried out by constant voltage with maximum of 5 mA per gel at 6 °C: 50 V for 30 min, then 100 V and 150 V for 30–30 min, reaching about two-third of the total running distance. At this stage, the blue cathode buffer (50 mM Tricine, 15 mM Bis–Tris, pH 7.0, 0.02% (w/V) Serva Blue G) was replaced by a cathode buffer without Serva Blue G, and the electrophoresis was followed by 200 V up to the removal of background stain for about 2 h. BN-PAGE was repeated two times with 4 samples/treatments.

To identify complexes, their polypeptide patterns were determined by a second-dimension SDS-PAGE according to Laemmlı (1970), modified by adding 10% (V/V) 87% glycerol to both stacking (5%) and separating (10–18% linear gradient) gels. 3-mm-wide gel strips were cut out from BN-PAGE lanes and were attached to the top of the denaturing gel in solubilising buffer containing 0.5% w/V agarose. Proteins were separated in the above-mentioned apparatus with a constant current of 20 mA per gel for 2 h at 6 °C. Following the electrophoresis, gels were stained according to Candiano et al. (2004).

Gels were scanned using an Epson Perfection V750 PRO scanner. Densitometry analysis (determination of pixel density on greyscale gel images) was carried out using the Phoretix image analysis software (Phoretix International, Newcastle-upon-Tyne, UK). Quantitative changes of Chl–protein complexes in the differently treated samples were assessed according to the pixel density of the different bands in the first-dimension BN lanes. Determination of spots was performed as in Sárvári et al. (2014).

Microarray Analysis

The total RNA samples were isolated from the second leaves of maize plants using the Direct-zol RNA Miniprep Kit (Thermo Scientific, Rockford, IL, USA), including the DNA digestion step. Quality and quantity of the extracted RNA were checked by a Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The samples were labelled with Cy3 (Low Input QuickAmp, Agilent), and 1650 ng cRNA was hybridised to the Agilent Whole Corn Gene Expression Microarray 4 × 44 K chip according to the manufacturer’s instructions (Agilent). The chip contained 42,034 probes for each sample, representing the whole maize transcriptome. The array was scanned using an Agilent Scanner, Extended Dynamic Range (100% and 10% laser intensities, 5 micron resolution). Signal intensities and normalisation processes were detected with the Agilent Feature Extraction (FE 9.5) and GeneSpring programmes (Agilent). Fold change (FC) values of the four samples were compared to each other with \( \log_{2}\text{FC} > |2| \) and \( P < 0.05 \) as follows: Co vs M, Co vs LT, Co vs MLT, M vs LT, M vs MLT. The functional annotation of the genes of interest was performed using Zea mays (Zm-B73-REFERENCE-NAM-5.0) and Arabidopsis thaliana (TAIR 10.1) sequences of the GenBank.

RT-qPCR Used for Validation of the Microarray and for Targeted Gene Expression Measurements

The same RNA samples of the microarray analysis were used for the validation of the microarray results and for further targeted gene expression measurements. cDNA was synthesised from 500 ng RNA with the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA), with the use of oligo(dT) primers. RT-qPCR reactions were run on an ABI StepOnePlus Real-Time PCR instrument (Thermo Fisher Scientific), using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Folylpolyglutamate synthase (FPGS), leunig (LUG) and membrane protein PBI10.07c gene (MEP) were used as internal control genes (Manoli et al. 2012) to normalise the Cq values of the genes of interests (Table S1). The geometric mean of the internal control data was applied for normalisation. The relative changes in gene expression were compared to the untreated control group and quantified according to the Pfaffl method (Pfaffl 2004). Primers (Table S1) were designed with the Primer3 online software (Koressaar and Remm 2007) and modified manually if necessary. Reaction efficiencies were calculated with the LinRegPCR software (Ramakers et al. 2003). The genes which expression were analysed by RT-qPCR are listed in Table S1. All genes used for the validation were chosen based on their significant expression changes reacting to the MMS pretreatment and the subsequent cold stress.

GO Analysis

GO analysis was carried out using the Cytoscape software (Shannon et al. 2003) and the BiNGO toolkit of the Cytoscape was used to describe and rank the gene functions into the three specific ontologies: biological process, molecular function and cellular component (Maere et al. 2005). All genes used for the validation were chosen based on their significant expression changes reacting to the MMS pretreatment and the subsequent cold stress.

Metabolomic Analysis

Total phenolic content was measured by a Lambda 25 UV/ VIS spectrophotometer (Perkin-Elmer, Foster, CA, USA) according to the method of Teklemariam and Blake (2004). 0.5 g fresh tissue of the second leaves was homogenised
in 10 ml methanol:water:hydrochloric acid (79:20:1, v/v) and centrifuged (10 min; 29,000 g). The supernatant was completed to 8.5 ml with the isolation mixture, and 0.5 ml Folin-Ciocalteu's reagent was added, and 3 min later, the samples were mixed with 1 ml saturated sodium carbonate. After 1 h, the reaction mixture was measured on 725 nm, and the results were represented in gallic acid equivalent (Teklemariam and Blake 2004).

HPLC methods were used for quantitative determination of polyamine and phenylpropanoid metabolites from leaves. In the case of polyamines, the measurement was carried out as described by Pál et al. (2013). 0.2 g samples of the 2nd leaves were homogenised with 1 ml 0.2 M perchloric acid, incubated on ice for 20 min, and after centrifugation at 10,000×g for 20 min, the supernatant was used. The polyamines were analysed via HPLC using a W2690 separation module and a W474 scanning fluorescence detector with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA). The HPLC separation of the plant extracts was performed on a Kinetex C18 column (Phenomenex, Torrance, CA, USA). For the determination of the phenylpropanoid metabolites, the second leaves and the basic 5 cm of the shoots were mixed, and 50 mg samples were homogenised with 1 ml methanol, incubated at 55 °C for 45 min, centrifuged at 10,000×g for 20 min and the supernatant was used for the further analysis. For chromatographic separation and high-resolution mass spectral analysis, a Dionex Ultimate 3000 UHPLC system was used hyphenated with an Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionisation (Thermo Fischer Scientific). Quantification of flavonoids was based on their HPLC–MS separation. The analytical performance characteristic of our HPLC–MS method was demonstrated by using different injected amounts of rutin standard. Linear regression analysis was performed, resulting in appropriate R² values. The amounts of other flavonoid compounds were calculated based on calibration with rutin.

**Statistical Analysis**

\( F_v/F_m \) was measured with 5 technical and 5 biological repeats, and the quenching parameters were measured with 3 technical and 3 biological repeats. The electrolyte leakage data consist of 9 replications of 3 biological experiments. The SOD, APX and GR activities were measured with 3 biological and 3 technical repeats. The thylakoid proteomic data contain 2 biological and 8 technical repeats. For the RT-qPCR experiments, 3 technical repeats and 3 biological repeats were used. HPLC measurements were carried out with 2 technical and 5 biological repeats. The results were statistically evaluated with ANOVA and Tukey’s honestly significant difference (Tukey’s HSD) post hoc test at 5% significance level \((P ≤ 0.05)\) using the RStudio programme package (Racine 2012). Microarray analysis was carried out with 3 biological repeats, and the results, after normalisation, were statistically evaluated by ANOVA at 5% significance level \((P ≤ 0.05)\). Principal component analysis (PCA) was used to test the similarity of the gene expression data sets of each pairwise comparison based on the significant log₂FC values \((P ≤ 0.05)\) of array probes. The centred and scaled log₂FC values were evaluated by `prcomp` and `cluster` packages and were visualised using ggfortify package in RStudio (Maechler et al. 2018; Tang et al. 2016).

**Results**

**Physiological Effects of the Treatments**

The first steps in the investigations were to study the interaction of cold treatment and MMS treatment by characterising the physiological condition of maize seedlings. Dynamics of the electrolyte leakage represents the changes of the membrane integrity, one of the earliest markers of cold damage (Fig. 1). The MMS treatment did not affect the membrane integrity compared to the control. However, in correlation with our previous observations, this value increases to cold treatment (Rácz et al. 2008). The application of MMS pretreatment greatly reduced the electrolyte leakage in MLT and maintained it on or approached to the level of Co.

Antioxidant enzyme activity measurements provide information on the ROS metabolic processes. Activity of six SOD isoforms was measured, localised in the following compartments: Mn SOD—mitochondrion and peroxisome; Cu–Zn SOD 1—chloroplast, Cu–Zn SOD 2—cytosol and cell wall, Cu–Zn SOD 3—peroxisome; Fe SOD 1—peroxisome, and Fe SOD 2—chloroplast. Only the chloroplast-localised Cu–Zn SOD isoform 1 showed significant increase in M, LT and MLT groups compared to the Co (Figs. S1, S2). The activity of chloroplast-localised Fe SOD 2 and the Mn SOD isoforms also elevated in LT and MLT, but not in a significant amount. The total enzyme activity of all measured isoforms was enhanced in each treatment, from which LT showed the highest value (Fig. S2). The APX activity showed no significant changes; however, M, LT and MLT groups had significantly higher GR enzyme activity (Fig. S3).

The \( F_v/F_m \) ratio represents the maximum quantum efficiency of PSII, providing information about the function of the photosynthetic electron transport chain as well. This ratio reliably decreases in several stress conditions, including chilling, thus is widely used for estimating sensitivity and tolerance against the actual environmental challenge in plants (Su et al. 2015). The MMS treatment did not change the \( F_v/F_m \) ratio compared to the control, while low temperature drastically decreased \( F_v/F_m \), to almost half of
the control values (Fig. 2). When MMS pretreatment was applied before chilling (MLT group), this decrease also occurred; however, MLT plants’ data were significantly higher than the values of LT. The quantum yield of PSII electron flow (ΦPSII) was not affected by MMS treatment, while significant changes could be observed in its values in the LT and MLT groups. Low temperature greatly decreased the level of PSII electron flow; however, MLT data have favourable mean values compared to LT. Thermal dissipation by non-functional PSII (ΦNF) strongly increased in both LT and MLT, but with a lower extent in MLT. While Φf,D increased to the chilling, ΦNPQ strongly decreased. There was no significant difference between these latter parameters in LT and MLT. Although the decrease in the total chlorophyll content is also a frequent concomitant of cold stress, in this case, there were no significant changes in total Chl or Chl a/b values either after 24-h cold treatment (LT) or in the other two treatments (M or MLT; Fig. S4).

Fig. 1 Effect of cold and MMS on the electrolyte leakage measured 1, 2, 3 and 4 h after the treatments. The values are given as a percentage of the maximal conductivity. Co—non-stressed control plants; M—MMS-treated plants; LT—plants exposed to 24-h cold stress; MLT—MMS-pretreated plants exposed to 24-h cold stress. Error bars represent standard deviation. Different letters indicate statistically significant difference (P ≤ 0.05; Tukey’s HSD).

Fig. 2 Maximum quantum efficiency of PSII (A) and photochemical quenching parameters (B) after MMS- and cold treatment. ΦPSII—quantum yield of PSII electron flow; Φf,D—fluorescence and constitutive thermal dissipation; ΦNPQ—light-dependent and ΔpH- and xanthophyll-mediated regulated thermal dissipation; ΦNF—thermal dissipation in non-functional PSII. Co—non-stressed control plants; M—MMS-treated plants; LT—plants exposed to 24-h cold stress; MLT—MMS-pretreated plants exposed to 24-h cold stress. Error bars represent standard deviation. Different letters indicate statistically significant difference for each parameter (P ≤ 0.05; Tukey’s HSD).
**Changes in the Organization of the Photosynthetic Apparatus**

Although the Chl content and Chl $a/b$ ratio of leaves and Chl $a/b$ ratio of mesophyll thylakoids (being around 3.9) did not show significant changes to the treatments, the results of the chlorophyll fluorescence measurements referred to strong functional differences in the photosynthetic apparatus under the different treatments. Thus, we decided to reveal the nature of the changes in the organization of thylakoids by BN-PAGE which is able to detect not only the variations in the quantity but also in the interactions of (macro)molecular complexes. The overall changes induced by the treatments are given in Figs. S5, S6 and Table S2. Amongst the pigment–protein complex forms, six showed significant differences in their amounts in 1D BN gels under the different treatments (Fig. 3). MMS treatment increased the amount of photosystem I-bound light-harvesting complex II subunits (PSI-LHCII). Cold induced a decrease in photosystem II-associated LHCII subunits (PSII supercomplex) and PSII-core dimer. At the same time, it enhanced the amounts of free LHCII assembly complexes (LHCII-a: moderately bound LHCII-trimers, associated with the CP29 and CP24 proteins) and Lhc monomers. Increase in the relative amount of Lhc monomers was more explicit in the MLT group. The variations in the amount of PSII-core dimers had a trend—decrease in LT and recovery in MLT—similar to that of Fv/Fm and $\Phi_{\text{PSII}}$ and opposite to that of the $\Phi_{\text{NF}}$ parameters. LT elevated the amount of CP43-less PSII cores, which have been present in even higher amount in the MLT group.

**Changes in the Gene Expression Pattern**

Linear regression analysis showed significant correlation between the microarray and RT-qPCR data ($R = 0.8421$) (Fig. S7). According to the microarray analysis, 4112 genes or gene variants showed at least twofold expression change in any experimental relation, meaning that approximately, 10% of the coding part of the genome was involved (Fig. S8, Table S3). The most genes reacted to the low temperature, while 591 genes proved to react clearly to the combined treatment (MLT). The MMS treatment per se did not significantly influence the gene expression pattern.

A principal component analysis (PCA) was carried out based on the significant log$_2$FC values of the microarray gene expression datasets in respect to each comparison. The result could help us to reveal the effects of MMS pretreatment prior to a low-temperature stress, denoting genes which expression change can contribute to the better physiological reactions of plants in the MLT group. We could identify two from the six principal components, which cover 93.78% of

![Fig. 3](image-url)
the variance, thus explain the majority of the data (Fig. S9). The first principal component (PC1) includes 66.64% of the data and has a moderate negative correlation with log2FC values of LT-M, Co-LT, M-MLT and Co-MLT comparisons (Fig. 4). The second principal component (PC2) includes 27.14% of the data and strongly correlates with the log2FC values of LT-MLT and Co-M comparisons. Three major groups of genes can be identified on the graph from which two is horizontally oriented and correlates with PC1. These three groups indicated by brown, green and blue dots refer to the significantly up- and down-regulated genes, thus showing the significant influence of chilling on the gene expression pattern. The effect of combined treatment supposed to be a smaller group of perpendicularly oriented genes indicated pink on the graph. The data correlate with PC2 and consist of a coherent group of up- and down-regulated genes scattered between the groups. According to the results PC1 and PC2 refer to chilling and MMS effect, respectively, what may help to clarify the genes of interest for further examinations.

Gene functions were analysed by GO annotation. The three custom independent ontologies constructed by Ashburner et al. (2000) were taken into consideration: biological process, molecular function and cellular component, from which the biological process category proved to be the most considerable and has been presented using the ClueGO software (Fig. 5, Fig. S10, Table S4). The graphic illustrates the major changes occurred in each treatment. We could identify five hits (photosynthesis; cellular amino acid, sulphur compound, and ROS metabolic processes; porphyrin and chlorophyll metabolism), that not only belong to the chilling stress response but may be the effect of MMS pretreatment on the gene expression pattern. The group of yellow dots is formed by genes related to generation of precursor metabolites and energy, photosynthesis, light-reaction, light-harvesting and pyridine-containing compound metabolic process. These GO groups contain 124 genes coding members of the antenna and light-harvesting complexes consisting of CP12, LhcA, LhcB, Psa and Psb genes. The group of brown and dark green dots is formed by genes involved in organic acid metabolic process, sulphur compound metabolic process, cellular amino acid metabolic process, pigment and cofactor metabolic process. These GO terms represent metabolic processes where SMM and SA expose their metabolic stimulation effects resulting in the change of the gene expression pattern. These groups contain a sum of 417 enzyme-coding genes, from which many take part in the phenylpropanoid, polyamine and porphyrin biosynthetic pathways (e.g. C4H, PAL, SAMS, FC, POR). We could identify numerous other GO groups that form a complex network and reveal a highly integrated regulatory system. These consist of response to hormone stimulus, response to ROS and oxidative stress. Moreover, we found genes taking part in the MAPK signalisation (MEK2) and transcription factors (WRKY, DREB, EIN, ARF). These modules regulate cellular and metabolic processes, thus stress response. This result provides a more detailed information and is in accordance with the PCA result, that also indicated the effect of MMS.

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 down-regulated in reaction to the applied treatments (Fig. 6, Fig. S11). The 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 down-regulated 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 twofold change, either activated or repressed. The changes were more pronounced in the MLT group. Amongst 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, pump and channel proteins and last but not least proteasome-related factors, showing that gene activation and stress response could also mean protein degradation as well. In the case of the chloroplast also numerous genes proved to be controlled, in the MMS-pretreated and cold-stressed plants (MLT) more than 100 genes were activated, coding for members of the photosynthetic apparatus and ATP synthase complex, nucleic acid metabolism-related proteins, members of the Calvin cycle, chlorophyll-binding proteins.
proteins, ABC transporters and the plastidic isoleucine-
tRNA ligase. Genes related to the plasma membrane were 
also represented in a relatively high number amongst 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 relevant 
genes, for instance members of the nucleic acid metabo-
lism (nucleotide kinases, helicases, PCNA, amongst others), 
transcription factors (G-box bZIP factors, bHLH Scream2, 
DREB1D, auxin-related factors, WRKY33), chromatin 
modifiers (histone deacetylase HDT2, lysine methyltrans-
ferase), 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 ini-
tiation factor isoform 4G-1, polyadenylation and cleavage 
factor homolog 4, SUMO1) (Table S5).

According to the PCA and GO analysis results, numer-
ous genes were selected for further investigation (Table S1). 
Genes involved in the metabolism of porphyrins (PRPO1,
FC1, FC2, POR1), carotenoids (ZXE), polyamines (ADC1, ADC2, ODC, SPDS, SPMS, SAMDC, SAMS) and the phenylpropanoid–flavonoid pathway (C4H, CHS, F3H, AGT), all related to cold acclimation, were taken into the expression analysis. The expression of ZXE was increased only in LT group (not shown). In the porphyrin metabolic pathway, MMS treatment enhanced the expression of POR1 and FC1 genes (Fig. 7). Chilling influenced PRPO, POR1 and FC1, moreover, a significant increase could be observed in MLT group except FC1. FC2 showed no significant changes in gene expression pattern. The relative gene expression was the highest in the MLT plants for all the studied genes of the porphyrin metabolic pathway.

The initial steps of polyamine biosynthesis are catalysed by the arginine and ornithine decarboxylases (ADC and ODC). The MMS treatment increased the expression of ADC1 and down-regulated ODC. Chilling stress only affected the expression of ADC2. The combined treatment stimulated ADC1, ADC2 and ODC, as well. Further steps of the pathway are catalysed by spermidine and spermine synthases (SPDS and SPMS). The expression of both genes remained unaffected in case of exogenous MMS. However, SPMS expression showed a slight decrease during chilling (LT) and the combined treatment (MLT). Propylamino groups, essential for the synthesis of SPD and SPM, are produced with the help of S-adenosylmethionine synthase and decarboxylase enzymes (SAMS and SAMDC). MMS increased the expression of SAMS, however, did not affect the expression of SAMDC. Chilling increased the expression of both genes, which was the most enhanced by the combined treatment MLT (Fig. 8).

Eight genes of the phenylpropanoid pathway were found to specially react to the treatments: phenylalanine ammonia-lyase (PAL), 4-Coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-dioxygenase (F3H), dihydrokaempferol 4-reductase (DFR), leucocyanidin oxygenase and anthocyanin 3’-O-glucosyltransferase (AGT). Each gene showed higher expression level in MLT group compared to LT. Four genes out of eight, from the initial, median and end section of the pathway (C4H, CHS, F3H, AGT), were further investigated (Fig. 9). MMS slightly enhanced the expression of CHS and F3H genes, in

Fig. 6 The result of GO analysis representing the number of cellular components-related genes that showed significant expression change. The number in parenthesis found after the GO group 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 changing expression data in the LT and MLT groups, related to the control. LT—plants exposed to 24-h chilling, MLT—MMS-pretreated plants exposed to 24-h chilling. The significant matches (P ≤ 0.05) are in white boxes. The grey boxes within the cell represent the non-significant matches, while the grey box outside the cell shows the number of genes with unknown localisation.
contrary had no effect on the expression of \(C4H\) and \(AGT\). Low temperature activated all genes. The combined treatment also increased the expression of all investigated genes, and, except of \(AGT\), invoked the highest expression.

**Changes in the Contents of Stress-Related Metabolites**

Concentration of putrescine (PUT) increased a little but significantly in M, and only a slightly and not significantly in MLT, while remained unchanged in LT (Fig. 10). Spermidine (SPD) and spermine (SP) were found in a higher amount than PUT and increased in M and MLT groups, resulting in a significant elevation in the level of SPM in both groups, while a significant increase of SPD in MLT plants and a non-significant increase of SPD in M group. The amount of 1,3-diaminopropane (DAP), a degraded polyamine, which process is catalysed by the PAO, slightly increased in reaction to chilling, but MMS had no effect on the concentration of this metabolite.

The highest concentration in the total phenolic content was measured in MMS-pretreated and then cold-stressed plants (MLT), while low temperature without MMS (LT) enhanced the metabolite level only a little and not significantly compared to the control (Fig. 11). According to the LC–MS results, 4 phenylpropanoid derivative compounds were identified, such as rutin (\(C_{27}H_{30}O_{16}\)), kaempferol-3-O-rutinoside (kaempferol, \(C_{27}H_{30}O_{15}\)), maysin (\(C_{27}H_{27}O_{14}\)) and neodiosmin (\(C_{28}H_{32}O_{15}\)) (Fig. 12). The most abundant metabolites are kaempferol-3-O-rutinoside and neodiosmin. The rutin concentration increased slightly, but not significantly in LT and MLT groups. We perceived higher kaempferol amount in every treatment compared to the control, from which the changes in the values of LT and MLT groups were significant. MMS had a minor effect on the concentration of maysin. The amount of neodiosmin decreased in both M and LT groups; however, level of MLT group remained the same as Co.

**Discussion**

Cold stress severely affects plant growth and development, especially those of the sensitive agricultural plants, which defence mechanisms against the stressor and acclimation processes are a key question. Biologically active compounds, like SMM, SA or the combined compound MMS are able to decrease stress damages (Gondor et al. 2016; Janda et al. 2018; Páldi et al. 2014). The protective effects of these naturally occurring substances are evidenced in several studies.
In the present work, we examined the mechanism of action of the exogenously applied MMS against chilling stress.

Plant physiological measurements were applied to characterise changes caused by the interaction of MMS pretreatment and cold stress. The electrolyte leakage measurements indicate membrane protective effects of MMS in case of chilling. This is similar to the membrane protective ability of the parent compound SMM (Rácz et al. 2008), though membrane stabilisation is observed for SA as well, for instance in case of salinity or in the absence of oxidising effects.
(Rivas-San Vicente and Plasencia 2011; Stevens et al. 2006). The decreased quantum efficiency of PSII and change in the quenching parameters are usual symptoms during cold stress (Allen and Ort 2001; Oláh et al. 2018). It is also proved, that SMM-pretreated plants had higher \( F_v/ F_m \) values compared to cold-stressed plants, thus the substance can help to maintain the relatively high photosynthetic activity (Páldi et al. 2014). SMM and SA trigger the biosynthesis of polyamines, phenolics and other stress-protective substances that contribute to the preservation of the photosynthetic membrane integrity (Hayat et al. 2010; Kósa et al. 2011). It is evidenced as well that SMM-, SA- or MMS-pretreated wheat plants had better photosynthetic parameters during salt stress, such as higher maximal and actual quantum yield of PSII (Janda et al. 2018). Our results also indicate that MMS pretreatment alleviates the damages of the plasma membrane improving the photosynthetic parameters, thus causing a better physiological condition during chilling stress.

Changes in the photosynthetic apparatus were also presumed according to the chlorophyll \( \alpha \) fluorescence measurements, which was evidenced by proteomics on the mesophyll thylakoid extracts. The significantly higher relative amount of PSI-LHCII complex in M shows major increase in the activity of the cyclic electron transport, which may result in the state transition of the grana. The pre-stress state transition can save the photosynthetic apparatus from exceed amount of light, thus alleviate the damages caused by the presence of a stressor. In case of SA treatment, stomatal closure can cause \( \Delta \text{pH} \) stimulation and ROS increase, which in turn can also induce state transition (Rochaix 2011; Janda et al. 2012). According to our measurements on the antioxidant enzymes, the plastid-localized Cu–Zn SOD and GR had enhanced activity in the M, LT and MLT groups, compared to the control, which may be caused by the increased ROS level in the chloroplast, thus confirm this assumption. Polyamines, such as SPM, were also reported to be able to conjugate LHCII, which may alleviate the damages caused by stress. Moreover, PAs due to their polycationic nature can stabilize PSII structure through electrostatic interaction. Through this interaction, PA treatment at low concentration can preserve PSII activity (Hamdani et al. 2011). The favourable \( F_v/F_m \) and \( \Phi_{PSII} \) values in context with the lower \( \Phi_{NF} \) parameter show more functional PSII, which was confirmed by the proteomic result. The CP43-free complex contains damaged D1 protein, that is targeted for degradation, then a new copy is synthesized resulting in the reassembly of PSII into the C2S2M2 holocomplex (Koochak et al. 2018). The C2S2M2 holocomplex contains a PSII-core dimer (C2), two strongly bound LHCII-trimers (S2) both attached to the core dimer by the CP26 protein and two moderately bound LHCII-trimers (M2) both attached to the core dimer by CP29 and CP24 proteins. Thus, the higher amount of the CP43-less PSII cores and Lhc monomers shows enhanced

Fig. 9 Relative expression changes of genes involved in the phenylpropanoid–flavonoid biosynthetic pathway. The expression of the examined genes is compared to the controls (Co), whose values are equal to one in each case. PAL phenylalanine ammonia-lyase, C4H cinnamate 4-hydroxylase, CHS chalcone synthase, AGT anthocyanin 3′-O-glucosyltransferase, Co—control, M—MMS-treated plants harvested 24 h after MMS treatment, LT—plants exposed to 24-h chilling, MLT—MMS-pretreated plants exposed to 24-h chilling. Values are means, ± SD is not shown (\( n = 3 \)). Different letters indicate significant difference (\( P \leq 0.05; \text{Tukey’s HSD} \))
repair mechanisms in MLT. It can be concluded that changes in the photosynthetic apparatus caused by MMS treatment give rise to more efficient photosynthetic processes during cold stress, which contributes to the better physiological parameters.

According to the favourable physiological parameters, it can be presumed that cold stress and especially the combined treatment had considerable influence on the gene expression pattern. Further details were revealed via microarray gene expression analysis. By applying PCA on the fold change values, we could identify a group of genes, which was considerably influenced by the combined treatment. More information was gained through the usage of GO annotation. The most pronounced expression pattern changes were in cellular components and metabolic processes after the combined treatment, compared to the cold-treated plants. In the work of Gondor et al. (2016), studying the effects of SA on the gene expression pattern, cellular process, metabolic process, response to stimulus and response to stress was amongst the most strongly affected GO categories, followed by
biosynthetic processes and macromolecule metabolism. In our study, major differences were observed in the number of up- and down-regulated genes in the plastid, the cytoplasm, the nucleus and the plasma membrane. As a result, there will be changes in the perception of stressor, regulation of stress response, as well as in the operation of photosynthetic apparatus, and in the photosynthetic activity. In addition, as a defence response, transport mechanisms and biosynthesis of stress-protective metabolites (e.g. polyamines, phenolics and flavonoids) will also be modified.

The nuclear PRPO1 gene encodes the plastid-localised protein PRPO that takes part in the porphyrin metabolism catalysing the protoporphyrinogen IX $\rightarrow$ protoporphyrin IX conversion. Protoporphyrin IX is a key precursor to heme and chlorophyll biosynthetic processes (Dayan et al. 2018). Our data show the most enhanced protoporphyrin synthesis in the MLT group, indicating increased biosynthesis of porphyrins in these plants; however, higher chlorophyll contents were not experienced. The elevated MLT POR1 expression would also indicate an activated chlorophyll synthesis. The protein expressed from FC1 is localized in both the plastid and the mitochondria; however, FC2 is a plastidial protein (Woodson et al. 2011). These two genes have a similar expression trend to POR1, but on a much lower level. The result indicates that the combined treatment more likely affected the plastidial, rather than the mitochondrial tetrapyrrole biosynthetic pathway. This should also imply a higher chlorophyll amount; however, no major changes were measured in the chlorophyll content and chlorophyll $ab$ ratio of the treatments. The enhanced chlorophyll metabolism in MLT may be the part of the repairing mechanism of the photosynthetic apparatus discussed above, where the damaged chlorophyll-containing proteins are replaced with functional ones. This process is relatively slow, so it is possible that the activity of degradative and synthetic processes counteracts each other. Thus, the dynamically changing chlorophyll content shows a relative stable level in the LT and especially in MLT groups.

In relation to the polyamine metabolic pathway, the PA biosynthesis genes tended to show maximal expression in the MLT group (as for ADC1, ADC2, ODC, SAMS and SAMDC), with the exception of SPD5 and SPMS, which expression did not change significantly. One or two ADC genes have been reported in plants, two paralogs, for instance, in Arabidopsis (Alcázar et al. 2011), tomato (Upadhyay et al. 2020) or maize (Gao et al. 2021), while only one in certain Citrus species (Song et al. 2022). ADC genes are shown to be cold-induced in the above-mentioned species; however, ADC1 seems to react stronger than ADC2. Szegő et al. (2009) showed that expression of ADC1 increased quickly in maize seedlings during a 24-h cold shock, but they did not examine ADC2. In our research, both paralogs showed increased expression to cold, not significantly for ADC1 and significantly for ADC2. Both genes reached their maximal expression in the MLT treatment. ODC gene expression level was induced only in the combined
treatment. This result suggests that MMS induces the initial steps of the polyamine biosynthetic pathway. Salicylic acid takes part in the signalling of abiotic stress and transcriptionally regulates polyamine synthesis (Janda et al. 2017), where a key regulator is a MAP kinase (MEK2). This process results in an enhanced putrescine level, which is confirmed by our HPLC data in the MMS-treated groups (M and MLT). According to the HPLC results of polyamine metabolites, the putrescine level was lower compared to the long-chain derivatives. Probably, the molecule transforms into spermidine and spermine, which may give more effective defence response during cold stress (Gondor et al. 2016). This HPLC result runs contrary to RT-qPCR data, where we measured low \textit{SPDS} expression compared to the control group. It may be a consequence of the periodic change of gene expression caused by the circadian clock of the plants. Proved or possible circadian regulation of spermidine synthesis has been found in mice (Zwighaft et al. 2015), fungi (Katagiri et al. 1998) and even in Arabidopsis (Kasukabe et al. 2004; Sanchez-Villarreal et al. 2013). However, the effect could also be caused by posttranslational regulation of enzyme activity, which is not connected with gene transcription. It is also possible that SPD and SPN contents are sufficient and no additional upregulation of their synthesis occurs. PUT content could also be upregulated later, providing a pool for further metabolism to SPD-SPN or to breakdown. On the other hand, it is possible that another \textit{SPDS} gene is responsible, rather than the ones occurred in our microarray dataset. The changes in the amount of DAP infer that the polyamine degradation does not belong to the main process of stress response at the presence of the stressor.

Phenolic compounds, such as flavonoids, help the acclimation of plants to the changing environmental factors through their scavenger role (Hernández et al. 2009). Anthocyanins are able to quench the exceeded light energy and protect the photosynthetic apparatus (Hernández and Van Breusegem 2010). These molecules also have antioxidant functions, which could be important in the acclimation processes as well (Treutter 2006). Our results provide information how chilling and MMS influence the gene expression patterns on the phenylpropanoid pathway, thus the amount and abundance of phenolics. LC–MS analysis provided details about the abundance of the phenolic-derivative flavonoids. The most abundant metabolites prove to be kaempferol-3-O-rutinoside and neodiosmin. The changes in the metabolite content and abundance show that MMS pretreatment has a stimulative effect on the pathway, which can be linked to the fact, that polyamines and SA also have an elevating impact on the phenylpropanoid synthesis (Chen et al. 2006; Wen et al. 2008). Due to their scavenger properties, these molecules can also contribute to the better value of PSII maximum quantum efficiency, thus promoting a favourable physiological state of the MMS-pretreated plants.
Conclusion

Our data show how the MMS combines the effect of SMM and SA on molecular level, thus causing numerous changes in the gene expression pattern and metabolite content, which can be linked to either one or the other compound. SMM acts as a substrate or stimulates other metabolic pathways through its derivatives. Additionally, SA, as a signal molecule, helps to fine tune the stress response; moreover, also stimulates metabolic processes. The exogenous application of SA increases the endogenous level of SA (Horváth et al. 2007), which can also influence the perception of SA signal. SMM and SA may cooperate mainly through the polyamine metabolism and influence the phenylpropanoid biosynthetic pathway (Fig. 13) (Pál et al. 2015). Based on the previous studies (Janda et al. 2018; Oláh et al. 2018) and our results, the MMS pretreatment induces priming mechanisms, through which it can alleviate the negative effects and prevent severe damages caused by chilling and salt stress in maize. This biologically active compound gives rise to a better physiological condition, thus it could provide an alternative, environmental friendly way to enhance the plants defence mechanisms against stressors. As MMS is more stable than SMM, it promises easier, more long-lasting and more cost-effective usage in agriculture, with a complementing effect of SA.

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Declarations

Conflict of interest  On behalf of all authors, the corresponding author states that there is no conflict of interest.

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