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BRIEF COMMUNICATION

S-methylmethionine alleviates the cold stress by protection of the photosynthetic apparatus and stimulation of the phenylpropanoid pathway

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Abstract

Maize (*Zea mays* L.) seedlings were pretreated with 0.001 g dm^{-3} S-methylmethionine (SMM) in the nutrient solution for 24 h and then subjected to chilling (6 °C for 2, 4, 6, 10, and 24 h). Cold stress significantly decreased the maximum quantum yield of photosystem II (variable to maximum chlorophyll fluorescence ratio, F_v/F_m) during the whole experiment but SMM pretreatment significantly reduced this decline. Content of phenolics and anthocyanins increased in response to low temperature, and SMM pretreatment further intensified the synthesis of these protective agents. These findings were supported by increased expression of genes coding enzymes of the phenylpropanoid pathway leading to synthesis of cinnamate-4-hydroxylase (C4H) and chalcone-synthase (CHS). Our results indicate that SMM pretreatment alleviates the low temperature stress by reducing the damage of the photosynthetic apparatus and stimulating the phenylpropanoid pathway.

Additional key words: anthocyanins, *C4H*, chilling, *CHS*, gene expression, phenolics, photosynthetic efficiency, stress defense, *Zea mays.*

Due to its subtropical origin, maize (Zea *mays* L.) is known for its high sensitivity to low temperature stress (Christie *et al*. 1994). Chilling has an effect on membrane fluidity, enzyme kinetics, and many other cellular processes. Moreover, it enhances the production of reactive oxygen species (ROS) resulting in damage to membrane functions, destabilization of protein complexes, and reduction of photosynthetic efficiency (Mahajan and Tuteja 2005).

Besides the transgenic methods aimed at improving stress tolerance (Belintani *et al*. 2012, Movahedi *et al*. 2012, Zhu *et al*. 2012), in recent years, increasing attention has been paid to compounds involved in plant defense mechanisms (Fu *et al*. 2011, Zhang *et al*. 2012). S-methylmethionine, a naturally occurring, biologically active non-proteinogenic free amino acid [$(CH_3)_2$-S-$(CH_2)_2$-CH(NH_2)-COOH] is such a compound (Rácz *et al*. 2008). It was discovered in cabbage (McRorie *et al*. 1954) but later studies revealed that it is universal in the plant kingdom. In plants, SMM is synthesized from methionine and can be reconverted resulting in a circular pathway known as SMM-cycle (Ranocha *et al*. 2001). SMM plays a role in the regulation of methionine and S-adenosylmethionine content (Mudd and Datko 1990), it is involved in the methylation processes in cells (Ranocha *et al*. 2001), and it is a key compound for the transport

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Abbreviations: C4H - cinnamate-4-hydroxylase; CHS - chalcone synthase; DMSP - dimethylsulphoniopropionate; EDTA - ethylenediaminetetraacetic acid; F_v/F_m - variable to maximum chlorophyll fluorescence (maximum quantum yield of photosystem II); GAE - gallic acid equivalents; MaizeGDB - Maize Genetics and Genomics Database; MEP - membrane protein gene *PB1A10.07c*; PAL - phenylalanine ammonia-lyase; PPFD - photosynthetic photon flux density; PS II - photosystem II; qRT-PCR - quantitative real-time polymerase chain reaction; ROS - reactive oxygen species; SMM - S-methylmethionine.

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and storage of sulfur (Bourgis *et al.* 1999).

Due to the complexity of its functions, SMM plays an important role in moderating the damaging effects of numerous stressors, such as low temperature (Gyetvai *et al.* 2002). SMM is a direct precursor of certain S-containing compounds involved in defense mechanisms (Kocsis *et al.* 1998) and also influence the biosynthesis of polyamines and ethylene (Ko *et al.* 2004, Rácz *et al.* 2008). In addition, it increases the stability of membranes and reduces the electrolyte leakage during cold stress (Gyetvai *et al.* 2002, Rácz *et al.* 2008). SMM has been successfully applied as a protecting agent against abiotic (Rácz *et al.* 2008, Szegő *et al.* 2009, Kósa *et al.* 2011) and biotic (Ludmerszki *et al.* 2011) stresses.

Chilling is often accompanied by oxidative stress, since low temperature increases the accumulation of ROS (Prasad *et al.* 1994, Kayihan *et al.* 2012). ROS are detoxified by antioxidative enzymes and low molecular antioxidants including different phenolic compounds (Pourcel *et al.* 2007). Content of phenolics can significantly increase in many stress conditions including low temperature (Christie *et al.* 1994, Chalker-Scott 1999, Winkel-Shirley 2002, Pourcel *et al.* 2007, Agati and Tattini 2010).

Plant phenolic compounds are synthesized through the phenylpropanoid pathway. In its second step cinnamic acid is converted to 4-coumarate by cinnamate-4-hydroxylase (C4H). C4H is a cytochrome P_{450}-dependent monooxygenase and it is essential for plant survival (Fraser and Chapple 2011). 4-coumaroyl-CoA, derived from 4-coumarate, is an important intermediate as a key point for divergence in the pathway. In the first branch, chalcone synthase (CHS) catalyzes the synthesis of chalcone by connecting 4-coumaroyl-CoA with three acetate units from malonyl-CoA (Fraser and Chapple 2011) further leading to the synthesis of flavonoids or anthocyanins. In the second branch, lignin is formed. The whole phenylpropanoid pathway is highly responsive to different abiotic stresses, such as wounding, drought, and low and high temperatures (Christie *et al.* 1994, Chalker-Scott 1999, Winkel-Shirley 2002) and it is also important in plant development (Souza Mello *et al.* 2012).

In the present work, we studied chlorophyll *a* fluorescence parameter F_v/F_m, content of phenolic compounds and anthocyanins in leaves and stems, respectively, and relative expression of *C4H* and *CHS* genes encoding enzymes involved in the phenylpropanoid pathway. As improving the tolerance of crops against chilling is a current challenge, the aim of this study was to present the beneficial effects of the naturally occurring, biologically active SMM on cold stress tolerance of maize.

Seeds of maize (*Zea mays* L. cv. Norma) were provided by the Agricultural Institute of the Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest. After germination, uniform 3-d-old seedlings were transferred to modified ¼ strength Hoagland solution containing 80 µM Fe in the form of EDTA complex. Plants were grown for 7 d in a growth chamber (*PGV-36*, *Conviron*, Winnipeg, Canada) under photosynthetic photon flux density (PPFD) of 250 µmol m^{-2} s^{-1}, a 14-h photoperiod, temperature of 23 °C, and relative humidity of 70 %. Nine days old plants were randomly divided into four groups. The non-stressed control (CO) and the low-temperature-stressed (LT) plants were kept at the original conditions, whereas the SMM-treated (SM), and SMM-treated and low-temperature-stressed (SL) plants were placed for 24 h in ¼ strength Hoagland solution containing 0.001 g dm^{-3} SMM. After one day, the SMM-containing solution was replaced by normal nutrient solution. Finally, 10-d-old plants of the cold stressed groups (LT and SL) were subjected to chilling stress under constant temperature of 6 °C for 2, 4, 6, 10, and 24 h. All measurements were performed on the 10th day after germination.

Changes in the maximum quantum efficiency of photosystem II (PS II), represented by the variable to maximum chlorophyll fluorescence ratio (F_v/F_m), were measured using a *PAM-2000* fluorometer (*Walz*, Effeltrich, Germany). Intact second leaves were measured individually after 15 min of dark adaptation under the given growth temperature. The maximum fluorescence was determined using a 0.8 s flash of white light with a PPFD value higher than 3 000 µmol m^{-2} s^{-1}. The standardized nomenclature presented by Van Kooten and Snel (1990) was used.

For determining the UV-absorbing phenolics and anthocyanin, 0.5 g fresh tissue from the second leaves or stems was extracted in methanol:water:hydrochloric acid (79:20:1, v/v) and the samples were cleared by centrifugation at 16 000 *g* for 10 min (Teklemariam and Blake 2004). As different phenolic compounds have different absorption characteristics within the UV range, changes in phenolic content were estimated by quantifying absorbance of the methanol extract in the following wavelengths: 254, 270, 290, 300, and 330 nm. A standard curve was prepared using gallic acid (GA) and the phenolic content was expressed as GA equivalents (GAE; Teklemariam and Blake 2004). Anthocyanin content of stems was measured by spectrophotometric analysis of 20-fold diluted methanol extracts at an absorbance of 530 nm (Christie *et al.* 1994) and was expressed as equivalents of cyanidin-3-glucoside (C3GE) based on its molar absorptivity (Lee *et al.* 2005). All the measurements were carried out with a *Lambda 25* UV/VIS spectrophotometer (*Perkin-Elmer*, Foster, CA, USA).

Leaf tissue mRNA was isolated with *GenoPrep* mRNA beads (*GenoVision*, Oslo, Norway) from second leaves according to the manufacturer's instructions. Extracted mRNA was treated with RNase-free DNase I. The first cDNA strand was synthesized from 0.5 µg of mRNA using *RevertAid First Strand* cDNA synthesis kit (*Thermo Scientific*, Rockford, IL, USA) and quantitative real time polymerase chain reaction (qRT-PCR) was performed using an *ABI PRISM® 7000* sequence detection system (*Perkin-Elmer*) with *Power SYBR® Green* PCR master mix (*Perkin-Elmer*). Primer sequences were designed using *Primer3* software (Rozen and Skaletsky 2000; Table 1). All cDNA samples were analyzed in triplicate and the cDNA was derived from two sets of independent experiments. Thermal cycling conditions of qRT-PCR consisted of 40 cycles of 95 ºC for 15 s, 60 ºC for 15 s, and 72 ºC for 30 s. A maize *actin* gene and the membrane protein gene *PB1A10.07c* (*MEP*) were included in the assays as internal controls for normalizing the variations in cDNA amounts used (Manoli *et al.* 2012). The geometric mean of the internal control data was applied for normalization. Genes of interest were *cinnamate-4-hydroxylase* (*C4H*) and *chalcone synthase* (*CHS*) (Table 1). The relative changes in gene expression were quantified according to the modified $2^{-\Delta\Delta CT}$ method of Pfaffl (2001), where calculated reaction efficiency levels (E) are taken into account.

The results were evaluated using analysis of variance (*ANOVA*), and the Student *t*-test was used for relevant data by *IBM® SPSS®* statistic software (*v. 20*). Significances of differences were determined at α values of 0.05, 0.01, and 0.001. Three biological and four technical repeats were performed for each experiment.

The chlorophyll *a* fluorescence parameter F_v/F_m, showing the maximum quantum efficiency of PS II, is widely used as a screening parameter for stress response and general physiological status (Lichtenthaler and

Table 1. qRT-PCR primers used for analyzing gene expression changes. Databases for accession numbers: *GenBank, **MaizeGDB. The PCR annealing temperature of all primers was 60 °C.

Gene	Accession number	Forward and reverse primer sequences (5' to 3')	Amplicon length [bp]	Reaction efficiency
Actin	J01238*	CGCTAGTGGGCGAACAACT and CGCATGAGGAAGTGTGTATCC	92	1.983
MEP	AY105120.1**	TTCCTCATGTTCTTCGTGCC and CAGTTCTCATTCCATCCGTG	130	1.994
C4H	BT039467*	GCGGAAGAAGGTGATGGCTC and AGAGGAGGTTGTCGTGGTTGAT	99	1.986
CHS	NM_001148774*	GCCGACTACCCGGACTACTA and CTTGCGGATCATCGACTTGT	102	1.990

Table 2. Changes in the maximum quantum efficiency of PS II (F_v/F_m), phenolic content [μmol(GAE) g^{-1}(f.m.)], anthocyanin content [μmol(C3GE) g^{-1}(f.m.)], and relative expression of *C4H* and *CHS* in maize seedlings. CO - control plants, SM - treated with SMM without cold exposure, LT - exposed to low temperature, SL - SMM-pretreated followed by low temperature exposure. Means ± SD, *n* = 4. Different letters within a row indicate significant difference at $P < 0.05$ (*), < 0.01 (**), and < 0.001 (***).

Parameter	Time [h]	CO	SM	LT	SL
F_v/F_m	2	0.74±0.01 ^{a}	0.77±0.00 ^{a}	0.70±0.00 ^{b} ***	0.73±0.01 ^{b} ***
	4	0.76±0.01 ^{a}	0.76±0.00 ^{a}	0.69±0.01 ^{b} ***	0.71±0.01 ^{b} ***
	6	0.74±0.00 ^{a}	0.75±0.01 ^{a}	0.70±0.01 ^{b} ***	0.70±0.02 ^{b} *
	10	0.75±0.02 ^{a}	0.75±0.01 ^{a}	0.65±0.01 ^{b} ***	0.68±0.01 ^{b} ***
	24	0.75±0.00 ^{a}	0.76±0.01 ^{a}	0.45±0.00 ^{b} *	0.60±0.02 ^{c} ***
Phenolic content	2	191.4 ±17.74 ^{a}	209.7 ±10.85 ^{a}	200.4 ±7.10 ^{a}	182.3 ±2.66 ^{a}
	4	187.7 ±10.49 ^{a}	196.5 ±17.33 ^{a}	242.3 ±23.15 ^{b} *	231.1 ±23.40 ^{b} *
	6	194.8 ±16.00 ^{a}	211.6 ±17.44 ^{a}	212.6 ±11.39 ^{a}	226.7 ±5.99 ^{a}
	10	178.1 ±13.17 ^{a}	193.2 ±13.66 ^{a}	274.5 ±8.79 ^{b} **	292.9 ±21.36 ^{b} *
	24	209.5 ±5.26 ^{a}	216.6 ±1.00 ^{a}	272.2 ±22.75 ^{b} *	302.1 ±9.68 ^{b} **
Anthocyanin content	2	17.49±1.75 ^{a}	15.74±0.58 ^{a}	20.70±1.17 ^{a}	23.62±1.17 ^{a}
	4	17.49±2.04 ^{a}	19.24±0.58 ^{a}	20.12±2.33 ^{a}	20.99±3.21 ^{a}
	6	16.91±0.29 ^{a}	17.49±2.92 ^{a}	24.49±4.08 ^{a}	28.86±2.92 ^{a}
	10	19.83±1.17 ^{a}	20.99±2.04 ^{a}	24.78±0.58 ^{a}	29.15±4.08 ^{a}
	24	24.78±1.75 ^{a}	25.07±4.08 ^{a}	37.61±0.87 ^{b} **	32.94±2.33 ^{a}
C4H	2	1.00	0.43±0.07 ^{a}	1.11±0.13 ^{a}	3.08±0.41 ^{a}
	4	1.00	3.50±0.19 ^{a}	12.21±0.75 ^{b} ***	14.26±1.72 ^{b} ***
	6	1.00	1.64±0.13 ^{a}	4.07±0.67 ^{b} ***	10.86±2.64 ^{c} ***
	10	1.00	1.11±0.18 ^{a}	12.25±1.89 ^{b} ***	35.63±5.13 ^{c} ***
	24	1.00	5.33±1.39 ^{a}	24.26±3.88 ^{b} ***	69.07±10.76 ^{c} ***
CHS	2	1.00	0.56±0.09 ^{a}	0.67±0.08 ^{a}	0.92±0.087 ^{a}
	4	1.00	2.66±0.12 ^{a}	2.69±0.06 ^{a}	3.81±0.35 ^{b} ***
	6	1.00	1.15±0.08 ^{a}	2.87±0.43 ^{b} ***	2.65±0.67 ^{b} ***
	10	1.00	0.62±0.08 ^{a}	2.08±0.25 ^{b} ***	1.32±0.15 ^{c} ***
	24	1.00	4.26±0.69 ^{a}	5.69±0.92 ^{b} **	10.09±0.86 ^{c} ***

Rinderle 1988). Like other stressors, low temperature usually decreases F_v/F_m, especially in plants sensitive to cold (Hetherington *et al.* 1989). According to our results, even after only 2 h of chilling stress, maize seedlings showed decline of F_v/F_m (Table 2). By the 24 h long treatment, the reduction of F_v/F_m was more pronounced and the values dropped to 0.45. However, seedlings that received SMM before being subjected to low temperature (SL) showed significantly higher F_v/F_m values than only cold-stressed plants (LT) did indicating that SMM improved the photosynthetic activity and probably had a positive effect on the overall physiological status of the plants. Seedlings treated only with SMM (SM) and control plants (CO) exhibited stable F_v/F_m values fluctuating around 0.75. This shows that SMM alone does not influence the physiological status of maize considerably. Kósa *et al.* (2011) also found that SMM reduced the decrease in F_v/F_m values of maize exposed to 1 - 4 d of cold treatments. Their results indicate that positive effect of SMM is caused by the reduction of membrane damage and preserving the functionality of PS II reaction centers. According to our data, SMM could produce its favorable effect in a shorter time-course when applied as a pretreatment before cold stress, whereas SMM application does not affect the normal photosynthetic activity of the unstressed plants.

Phenolic compounds were more abundant in leaves whereas anthocyanins were more easily characterized from the stem where they are usually perceptible to the naked eye. The content of both UV-absorbing compounds increased in response to low temperature. The content of phenolics significantly increased after 10-h chilling, though a certain rise was recognized in plants subjected to 4 h of chilling as well (Table 2). When SMM treatment was applied, the phenolic content of the chilled samples increased even more. SMM did not significantly affect the phenolic content at control temperature. Changes in anthocyanin content in the stem were similar (Table 2). Cold exposure induced the biosynthesis of anthocyanins at all points of the 24 h time-course. With SMM pretreatment, a higher content of anthocyanins was measured after cold stress, except for 24-h chilling. When temperature was not lowered, SMM-treated and control plants accumulated practically the same amount of anthocyanins in their stems. It could be proved that SMM pretreatment enhances the responses to chilling resulting in elevated biosynthesis of the secondary metabolites. Christie *et al.* (1994) demonstrated that the general phenylpropanoid pathway is induced by cold stress in maize. The direct protecting role of flavonoids under stress was also proved by Pourcel *et al.* 2007. Hernández and Van Breusegem (2010) presumed that flavonoids, in addition to their well-known protective functions, act as energy escape valve during abiotic stresses. In our approach, SMM-induced increase of anthocyanin and phenolic content probably contributed to the reduced impact of cold stress on maize seedlings. Our present results confirm the data of Kósa *et al.* (2011).

SMM treatment in non-chilled plants caused only a slight fluctuation in *C4H* gene expression. The magnitude of the change remained remarkably lower than in cold-stressed plants indicating that the effect of SMM alone was slight as compared to the exposure to low temperature with or without a preliminary SMM treatment. Cold exposure raised the transcription rate after 4-h treatment, though a remarkable drop was detected in plants subjected to 6-h chilling, yet the difference of transcript levels between LT and SL groups remained significant. After this decline, a continuous increase was shown in the cold-stressed plants (Table 2). Seedlings having the SMM pretreatment reacted to chilling in a similar but more intensive manner: their *C4H* expression showed a smaller decrease between 4- and 6-h treatments and a faster increase between 6- and 24-h cold exposure up to a 69-fold peak as compared to the control. Except for 2- and 4-h treatments, the increase of the relative ratio of transcripts proved significant differences between LT and SL plants.

Changes in the expression of *CHS* were similar to those of *C4H* (Table 2). Low temperature resulted in some stimulation after 4 and 6 h with a little decrease at 10 h and a near 6-fold increase at the end of the 24-h period. SMM-pretreated plants reacted to chilling in a similar way with a 10-fold increase after 24-h cold treatment which is significantly higher than in chilled plants without the addition of SMM. SMM treatment at control temperature resulted in changes similar to those induced by chilling with the difference that expression rate values were lower after 6, 10, and 24 h of treatment (Table 2).

Genes encoding enzymes of the phenylpropanoid pathway react differently to chilling. Christie *et al.* (1994) found that *phenylalanine ammonia-lyase* (*PAL*) and *CHS* are among the genes induced by low temperature stress in maize. Cold-induced expression of *C4H* in citrus species was studied by Meng *et al.* (2008) who showed a 2.5-fold up-regulation in response to a moderate cold acclimation but did not find a reliable transcriptional induction after a stronger cold treatment. According to our results, *C4H* expression was much more increased by cold stress than *CHS*. This indicates a general activation of the phenylpropanoid pathway and not only the flavonoid-anthocyanin branch beginning with the reaction catalyzed by CHS. Nevertheless, compounds synthesized in this branch effectively participate in the stress response of chilled maize in eliminating ROS. SMM obviously increased the transcription of both *C4H* and *CHS* in maize exposed to low temperature showing a general inductive effect on the phenylpropanoid pathway.

The effect of SMM on the stress response of maize seedlings exposed to chilling temperature for various lengths of time were examined on three different levels. Overall physiological status was monitored by parameters of chlorophyll fluorescence, the effects of SMM on the expression of genes linked to stress response pathways were analyzed by qRT-PCR, and secondary metabolic changes were examined by measuring the content of phenolics and anthocyanins. Summing up these results, it could be stated that SMM pretreatment prevent the damage of the photosynthetic apparatus under cold stress and stimulate the phenylpropanoid pathway.

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