Volume 58(1):1-5, 2014
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

S-methylmethionine alters gene expression of candidate genes in *Maize dwarf mosaic virus* infected and drought stressed maize plants

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ABSTRACT In the present work we investigated the potential beneficial effects of the exogenous application of S-methylmethionine (*SMM*) that plays an important role in the plants' sulphur metabolism and contributes to the production of certain defence compounds. The possible beneficial effects were challenged against *Maize dwarf mosaic virus* infection and drought stress. We studied the expression changes of *GF14-6* and *SAMS* during viral infection and *DREB2A* and *DBP2* during drought stress. The product of *GF14-6* recognise the viral coat protein and contributes to RNA-silencing, while the product of *SAMS* plays a central role in the plant sulphur metabolism and contributes to the production of several defence compounds. The products of *DREB2A* and *DBP2* contribute to better plant defence against drought stress and increase the efficiency of water uptake. According to our results, SMM pretreatment has a considerable change on the investigated genes' expression. It significantly decreases the gene expression of *GF14-6*, while infection results in a higher expression level. 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. *SMM* lessens the gene expression of *DREB2A*, while no changes were observed in *DBP2* compared to drought stressed plants.
Acta Biol Szeged 58(1):1-5 (2014)

KEY WORDS

drought
gene expression
maize
Maize dwarf mosaic virus
S-methylmethionine

Maize (*Zea mays* L.) is one of the most widely cultivated crops worldwide. Apart from being a well-known food and feed plant, it also plays an important role in industry as well, therefore, a great emphasis is put on its research in order to maintain health and crop production.

Maize dwarf mosaic virus (MDMV) is one of the most important pathogens of cultivated sweet corn varieties. The infection usually causes crop losses of 10-45% (Oertel et al. 1997). MDMV preferentially colonizes members of the Poaceae family (such as *Z. mays*) and spreads via aphid, pollen and seed transmission (Tóbiás et al. 2007; Gell et al. 2010; Stewart et al. 2012). Though the precise molecular details of maize response reactions are largely unknown the enhanced expression of *S-adenosylmethionine synthase* (*SAMS*, GenBank: BT054969) and *GF14-6* (MaizeGDB: BG836057.1) (Uzarowska et al. 2009) are considered as typical answers in maize varieties. *SAMS* is important in the synthesis of S-adenosylmethionine, which molecule is important in cellular methylation processes and contributes to the synthesis of plant defence hormones and compounds (Mudd and Datko 1990). *GF14-6* is a member of the 14-3-3 protein family, and is capable of recognising parts of the viral coat protein therefore it has an important regulatory role during plant defence (Konagaya et al. 2004).

As sessile organisms, plants are constantly challenged by a wide range of abiotic stresses as well, from which drought can be mentioned. Drought affects more than 10% of land crops, resulting in more than 50% of crop loss worldwide (Lata and Prasad 2011). In the last decades severe droughts were experienced in the Carpathian basin, mainly due to global climate change, therefore it is becoming a crucial problem in agriculture. During drought stress the upregulation of *DREB2A* (MaizeGDB: AB218833) and *Zea mays DRE-binding protein 2* (*DBP2*, MaizeGDB: FJ805750) genes can be observed, which represent the two main molecular pathways activated during drought. *DREB2A* is present in an abscisic acid independent pathway (Sakuma et al. 2006), while on the other hand *DBP2* takes place in an abscisic acid dependent route and directly binds to DNA and exerts its effects (Zhang et al. 2011). Both of these genes are important in plant defence and are responsible for a more efficient water uptake and drought stress tolerance.

The use of biologically active compounds could be a feasible way of improving tolerance to certain abiotic and biotic stress factors. S-methylmethionine [SMM, $(CH_3)_2$-S-$(CH_2)_2$-CH(NH_2)-COOH], which occurs naturally in the plant kingdom as a non-proteinogenic, sulfur-containing amino

Accepted Aug 25, 2014
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Table 1. A list of primers used in the investigation, including the reference genes (*Z. mays actin - ZA*, *maize membrane protein PB1A10.07c* - MEP) and the genes of interest (*GF14-6*, *SAMS*, *DREB2A* and *DBP2*).

Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	qRT-PCR efficiency E
ZA	CGCTAGTGGGCGAACAACT	CGCATGAGGAAGTGTGTATCC	1.983
MEP	TTCCTCATGTTCTTCGTGCC	CAGTTCTCATTCCATCCGTG	1.994
GF14-6	AGAGCAATGTCCTGGGCAG	CAAGATGAAGGGTGATTACTAC	1.992
SAMS	CATTGAGCAGCAGTCCCCT	GGTCTCGTCAGTCGCATAC	1.987
DREB2A	GTGCTGTGGTGCATGGT	CGTAGGCCCATCTCGTGATC	1.991
DBP2	GCCCGATGGCATTTTAGACG	AACCAGGAGATTAGCACGCA	1.989

acid, could be used to enhance resistance. In plants, SMM is synthesized from methionine and can also be regenerated in the SMM-cycle (Mudd and Datko 1990). Similarly as S-adenosylmethionine, SMM is involved in the methylation processes taking place in the cytoplasm, and is an important compound in the transport and storage of sulphur (Bourgis et al. 1999). SMM contributes to improved plant resistance, as it is a direct precursor of the osmoprotectant sulfopropionates involved in defence mechanisms, while also influencing the biosynthesis of plant regulatory and defence compounds such as polyamines and ethylene (Ko et al. 2004).

The aim of this work was to reveal the gene expression changes following viral infection or drought stress both coupled with SMM treatment, by which means the protective effects of SMM can be demonstrated. The changes of *GF14-6*, *SAMS*, *DREB2A* and *DBP2* are evaluated here, whose products are all important in plant defence.

Materials and Methods

Plant growth conditions

Zea mays L. var. *rugosa Jubilee* (sweet corn) plants were grown on Hoagland solution of ¼ strength (containing 80 µM Fe(III)-EDTA as iron form) in growth chamber SANYO MLR-350 HT, with a 14/10 h light/dark period and a light intensity of 300 µmol photon m^{-2} s^{-1}, a day/night temperature of 25/22 °C and 70% relative humidity. To study the effects of SMM, 11-d-old plants were placed in Hoagland solution of ¼ strength containing 2 mg l^{-1} SMM for 24 h. MDMV infection was carried out on the 12th and 14th days of the treatment. The first and second leaves of the plants were inoculated mechanically with Dallas-A strain MDMV particles. Leaves from infected plants developing macroscopic symptoms were homogenized in Sörensen phosphate buffer (pH 7.2, 0.06 M, 0.3 mg dm^{-3}) and were used for inoculation. Carborundum was added as an abrasive. Gene expression changes of the investigated genes were measured 1, 2 and 3 weeks after the infections. To demonstrate drought stress on the 12th day of the treatment PEG-6000 was added to the Hoagland solution of ¼ strength (in 15% of concentration), and plants were grown on that medium for a 1-week period. The real-time PCR measurements were carried out during this 1-week period on the 1st, 3rd and 7th days.

Gene expression changes

qRT-PCR measurements were carried out using the Power SYBR® Green PCR Master Mix (Life Technologies, Foster, CA, USA). The experiments were run on an ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster, CA, USA). The primer efficiencies were evaluated by applying a standard curve, generated from five points of a tenfold dilution series. The slope of the standard curve was fitted into the following equation: $E = 10^{-1/slope}$, where E stands for the efficiency of the given primer pair. The results are indicated in Table 1. The relative changes in gene expression were quantified according to the modified ΔΔCt method of Pfaffl (2001), where E (efficiency) values are taken into account.

Statistical analysis

The results were evaluated using analysis of variance (ANOVA) with SPSS® statistical software (version 20, IBM®) followed by Duncan's multiple range test (DMRT) to test for significant differences between means at $P \leq 0.05$ (Duncan 1955). DMRT considers the pairwise Student's t test comparisons of different datasets. In DMRT letters of the alphabet show the significant differences, where letter 'a' indicates the greatest significantly different average value of a data group, 'b' stands for the second greatest, and so on. Mixed letters (such as 'ab') indicate that these data groups do not differ significantly from 'a' or 'b' values. Three biological and three technical repeats were performed for each experiment.

Results and Discussion

In the present work, the stress responses of maize plants infected with MDMV and exposed to drought stress were studied, and compared to the data of SMM-treated plants and untreated controls. The spread of MDMV was monitored over a three-week period after virus inoculation, while the effects of drought stress for one week.

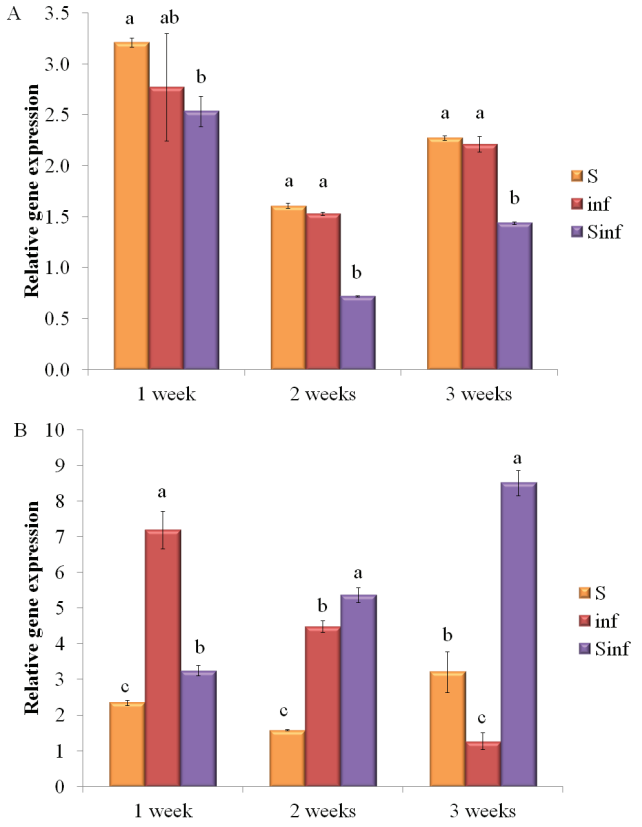

Figure 1. Changes in the relative gene expressions of *GF14-6* (A) and *SAMS* (B) of SMM-treated and MDMV-infected *Z. mays* leaves, shown in relate to control, meaning that the values of the control are equivalent to 1 unit of relative gene expression in all cases. Abbreviations: S - SMM-treated, inf - MDMV-infected, Sinf - SMM-pretreated followed by MDMV infection, *GF14-6 - G-box factor 14-6, SAMS - S-adenosylmethionine synthase*. Letters indicate significant differences at $P \leq 0.05$ according to DMRT. Letter 'a' indicates the greatest significantly different average value of a data group, 'b' stands for the second greatest, and so on. Mixed letters (such as 'ab') indicate that these data groups do not differ significantly from 'a' or 'b' values. Error bars represent standard deviations, n=9.

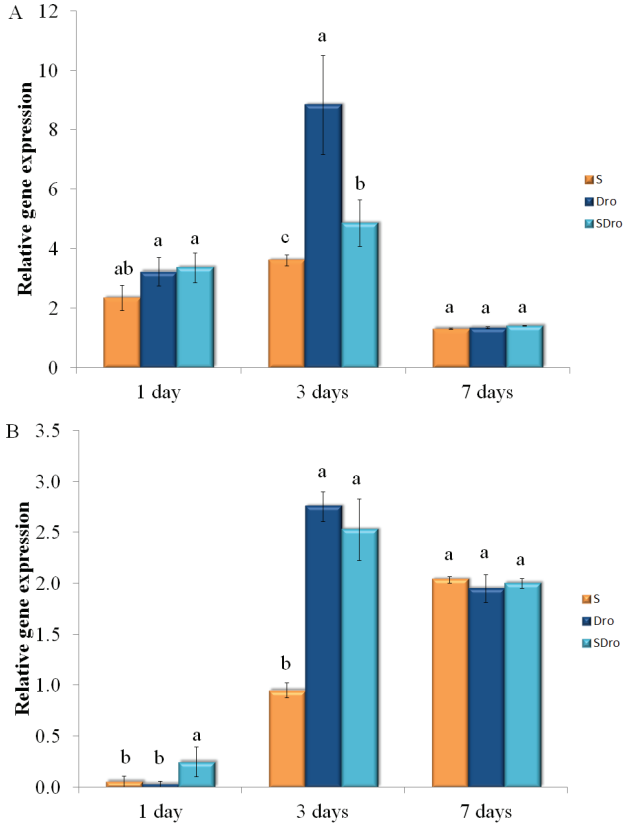

Figure 2. Changes in the relative gene expressions of *DREB2A* (A) and *DBP2* (B) of SMM-treated and drought-stressed *Z. mays* leaves, shown in relate to control, meaning that the values of the control are equivalent to 1 unit of relative gene expression in all cases. Abbreviations: S - SMM-treated, Dro - drought-stressed, SDro - SMM-pre-treated followed by drought stress. Letters indicate significant differences at $P \leq 0.05$ according to DMRT. Letter 'a' indicates the greatest significantly different average value of a data group, 'b' stands for the second greatest, and so on. Mixed letters (such as 'ab') indicate that these data groups do not differ significantly from 'a' or 'b' values. Error bars represent standard deviations, n=9.

In infected plants the mechanical effects of the rubbing (without virus particles), referred to as negative control were investigated previously. According to our results, in a short term period (1-3 days after the mechanical rubbing) stress response was detected, but only in the mechanically rubbed 1st and 2nd leaves. One week after the infections the harmful effects diminished, and when gene expression measurements were conducted from the younger leaves of negative control (4th to 6th leaves), the average values did not differ significantly from control plants.

During the course of MDMV infection significant changes occur in the plant metabolism, therefore in gene regulation as well. The gene expression levels of *GF14*-6 changed in a similar manner in all treated groups, since the 2.5-3-fold increase in the 1st week was followed by a decrease in the 2nd week and a subsequent elevation in the 3rd week (Figure 1A). The decrease was the most expressed in infected plants that were SMM-pretreated as well, whose *GF14-6* expression values decreased under the control level in the 2*nd* week, and were always exceeded by the two other groups, indicating a weaker stress response. Based on Konagaya et al. (2004), 14-3-3 proteins can recognize parts of the coat protein of *Tobacco mosaic virus*, contributing to RNA silencing. We assume that in the case of MDMV infection a similar mechanism may take place. Therefore, we propose 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 defense as a result of SMM pretreatment

MDMV infection caused a 7-fold rise in expression of

SAMS one week after the treatment (Figure 1B), though a continuous fall-off was found in the gene activity, approaching control level in the 3rd week. By contrast, in SMM-pretreated and subsequently infected plants the gene expression increased in a moderate rate with a continuous elevation, reaching a more than 8-fold enhancement (compared to control) in the 3rd week, indicating a less rapid but even long-lasting response to the MDMV infection. In only SMM-treated plants the expression of *SAMS* changed about 1.5-3-fold. These results suggest that this gene product play an important role during MDMV infection, probably by upregulating the formation of S-adenosylmethionine involved in numerous methylation processes, and therefore enhancing the SMM-cycle, further contributing to the production of defence compounds. SMM pre-treatment remarkably changed this profile. A more prolonged increase was observed compared to only infected plants, ensuring a constant long-lasting expression of this versatile enzyme involved in many aspects of gene transcription, cell housekeeping and secondary metabolite production. SMM treatment in uninfected plants slightly increased the expression of *SAMS*, presumably due to the contribution to the methionine and SMM-cycles.

Osmotic stress triggers either abscisic acid-dependent or abscisic acid-independent molecular pathways, resulting in an advanced plant defence. *DREB2A* is located in the abscisic acid-independent pathway in an upstream position, whereas *DBP2* is localised in the abscisic acid-dependent pathway and in a more downstream position (Sakuma et al. 2006; Zhang et al. 2011). Therefore a comprehensive picture is acquired with the expression study of these two genes about drought stress signalling. During the investigation 1-d, 3-d and 7-d stressed plants were studied. The gene expression changes of *DREB2A* were most pronounced in 3-d stressed plants (Figure 2A). In drought-stressed plants approximately a 9-fold increase was measured compared to control plants, while when SMM pretreatment was applied prior to drought stress, only a 4.5-fold increase was detected. Even in 1-d stressed plants a considerable increase is observed in the gene expression (around 3-fold increase in drought-stressed plants), due to the protein's upstream regulation. The results show that the product of *DREB2A* plays an important role in osmotic stress, its relatively early appearance support its importance in early plant defence. When SMM was given to drought-stressed plants a slighter increase was observed in the gene expression indicating a moderate activation of the ABA-independent pathway.

Slightly different results were acquired in the case of *DBP2* (Figure 2B). In 1-d stressed plants a significant decrease was found in all treated groups. 3-d stressed plants had a 3-fold increase compared to the control, irrespectively of SMM treatment prior to drought stress. After 7-d all treated groups showed an about 2-fold expression of the control. The data indicate that the abscisic acid-dependent pathway is also active during osmotic stress, but lower levels of gene expression show its less importance in this process. Also, SMM pretreatment resulted in no changes compared to drought stressed plants, indicating that SMM may not play such an important role in this pathway.

The results suggest that the gene products of *GF14-6*, *SAMS*, *DREB2A* and *DBP2* play a crucial role in the mechanisms of plant defence against MDMV infection and drought stress. The natural compound SMM has a beneficial effect on the stress response, resulting in an increase in the defence potential of maize plants during MDMV infection and drought stress. Furthermore we assume that similar results would be achieved while observing other biotic and abiotic stresses as well. These observations are in agreement with our previous findings (Rácz et al. 2008; Ludmerszki et al. 2011).

Acknowledgements

The authors thank Györgyi Balogh and Asztéria Almási for their technical assistance. This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/1-11-1-2012-0001 'National Excellence Program , and by the grant of the Hungarian Scientific Research Fund (OTKA 108834).

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