Theses of doctoral dissertation

The effects of S-methylmethionine and S-methylmethionine-salicylate in *Maize dwarf mosaic virus*-infected sweet corn

Made by: *Edit Ludmerszki*

Eötvös Loránd University of Science, Doctorate School in Biology

Prof. Dr. Anna Erdei

Experimental Plant Biology PhD Program

Prof. Dr. Zoltán Szigeti

Dr. Gábor Kovács M.

Supervisor:

dr. Szabolcs Rudnóy

Assistant Professor

Eötvös Loránd University of Science

Department of Plant Physiology and Molecular Plant Biology

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Introduction

Maize is one of the most widely cultivated crops worldwide. It is an important food and feed plant, therefore great emphasis is put on its research. *Maize dwarf mosaic virus* (MDMV) is a significant pathogen of maize varieties. Mosaic pattern appears on the leaves of infected plants, and a reduction in height and crop production occurs, thus the infection results in great agricultural losses. In the environment the virus spreads via aphid, pollen or seed transmission. Several ways exist, by which the virus can be restricted: with the use of insecticides the aphids can be killed, infected plants can be removed from the field, moreover, resistant plants can be used that are produced via genetic modification. In practice, however, none of these methods result in a 100% protection against MDMV-infection. Therefore, the need arises to introduce a method, by which the plants can be protected against the infection, furthermore it poses no hazard to the environment or to human health.

In my work I was searching for the question: What effects do S-methylmethionine (SMM) and S-methymethionine-salicylate (MMS) have on the defence system of maize plants? SMM is a natural compound, biologically active, non-proteinogenic amino acid, moreover, it is widely used as a human and animal medicine known as vitamin U to treat ulcer and other gastrointestinal diseases. MMS is a newly synthesized compound, which contains SMM and salicylic acid (SA); the latter molecule is a well-known compound, being an important component of anti-inflammatory medicines.

Objectives

- It was my aim to investigate the spreading of the virus within the plants and the changes in the amount of viral particles as a result to SMM- or MMS-treatments.
- It was my objective to monitor the changes in the chlorophyll content and the status of the photochemical system, in order to investigate the potential protective effects of the treatmens prior to infection.
- I investigated the activity of the pathway of the phenylpropanoid biosynthesis and the total phenolic content in different plant parts in order to varify the protective effects of SMM and MMS during MDMV-infection.
- I measured the activity and changes of the antioxidant system, to show the effects of the treatments in infected plants.

- I investigated the gene expression changes of *GF14-6* and *SAMS* as a consequence of SMM- and MMS-treatements. These two genes are known to be activated during MDMV-infection.

Materials and Methods

Applied treatments:

Sweet corn (*Zea mays* cv. *saccharata* var. Honey Koern., and var. Jubilee) were used. The plants were allocated into six groups according to treatments: control, SMM- or MMS-treated (*smm, mms*), MDMV-infected (*mdmv*), and SMM- or MMS-pretreated plants prior to infection (*smm+mdmv, mms+mdmv*). SMM- and MMS-treatments were carried out at 10 days of age in 0.5 mM concentration for 24 hours, while MDMV-infection was performed on 11 and 13 days old plants. Measurements were carried out 1, 2 and 3 weeks after the treatments.

Enzyme-linked immunosorbent assay (ELISA):

ELISA technique was applied to determine the amount of MDMV viral coat protein (Bioreba AG, Reinach, Switzerland). Samples were collected from the leaves and roots. Colour change (due to the enzymatic reaction) was measured at 405 nm on a Labsystem Multiscan MS spectrophotometer.

Determination of chlorophyll content:

To determine the chlorophyll content from the leaves, samples were homogenized in 80% aceton (containing ammonia), and were measured at 646.6, 663.6 and 730 nm on LAMBDA 25 UV-VIS spectrophotometer (PerkinElmer Life and Analytical Sciences, Sheltin, CT, USA). These values were used to determine the chlorophyll content of the plants.

The measurement of non-photochemical quenching parameters:

Chlorophyll fluorescence and non-photochemical quenching parameters were measured non-invasively with pulse amplitude modulation technique (PAM 101-102-103, Waltz, Effeltrich, Germany), following 15 minutes of dark adaptation.

Chlorophyll fluorescence imaging:

For chlorophyll fluorescence imaging FL-FIS (compact flash-lamp fluorescence imaging) system was used. The leaves were illumintaed with 335 nm UV-light, and the emitted fluorescence was detected at 440, 520, 690 and 740 nm with a CCD camera. For data evaluation the fluorescence values of 440 and 520 nm were used.

Determination of total-phenolic content with photometer:

Total-phenolic content was determined photometrically (LAMBDA 25 UV-VIS spectrophotometer, PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Samples taken from leaves and stems were homogenized in metanol-hydrochloric acid, and absorbance values were recorded at 254, 270, 290 and 300 nm. The total-phenolic content of the samples was calculated from the average value of the four distinct absorbance values and was determined in relation to gallic acid.

Description of the enzyme kinetics measurements:

In order to evaluate plant defence the activities of several antioxidant enzymes were monitored. Measurements were carried out on a LAMBDA 25 UV-VIS spectrophotometer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). The measured enzymes: ascorbate peroxidase, gluthation reductase and guaiacol peroxidase.

RNA-extraction and cDNA-synthesis:

For RNA-extraction ZR Plant RNA MiniPrep^{TM} 2024 (Zymo Research, Irvine, CA, USA) kit was used, according to the manufacturer's instructions. Samples were collected from leaves, stems and roots. For cDNA-synthesis RevertAid First Strand cDNA Synthesis (Thermo Scientific, Rockford, IL, USA) kit was used, according to the manufacturer's instructions. The expression of the following genes was investigated with quantitative real-time PCR (qRT-PCR): *actin, membrane protein PB1A10.07c, chalcone synthase (CHS), cinnamic acid 4-hydroxylase (C4H), GF14-6, S-adenosylmethionine synthase (SAMS), ascorbate peroxidase (APX), glutathione reductase (GR)* and *guaiacol peroxidase (GPX).* Apart from these measurements, MDMV viral RNA content was determined by absolute quantification qRT-PCR. Measurements were carried

out on an ABI StepOnePlus™ Real-Time PCR System machine (Life Technologies, Foster, CA, USA).

Results

Changes in the amount of MDMV:

According to my results, both SMM- and MMS-treatments resulted in a decrease in MDMV in all the investigated plant parts.

Changes in the chlorophyll content of SMM-treated infected plants:

In Jubilee sweet corn *smm+mdmv* plants had higher chlorophyll content 2 and 3 weeks after the treatments, than *mdmv* plants. Such changes were not observed in Honey sweet corn.

Changes in the non-photochemical quenching parameters due to the treatments:

A considerable decrease is observed in ΔpH-dependent quenching and photoinhibition in the third week of *smm+mdmv* and *mms+mdmv* plants. In *smm+mdmv* and *mms+mdmv* plants the state-transition dependent quenching is increased. As a result to SMM- and MMS-treatments there is an increase in the number of active PS II reaction centers, and a decrease is observed in the fluorescence and heat-dissipation pathways compared to *mdmv* plants.

Changes in the phenolics content of pretreated and infected plants:

In *smm+mdmv* plants an increase can be observed in the values of F440 and F520 compared to infected ones, except for the values of F520 on the 2nd week. Shortly after the treatments, there is a decrease in total phenolic content in *smm+mdmv* plants, while in *mms+mdmv* plants a slight increase can be observed. Both SMM- and MMS-pretreatments increase the gene expression of *cinnamic acid 4-hydroxylase* and *chalcone synthase* compared to *mdmv* plants.

Changes in the activity and gene expression of antioxidant enzymes:

Changes of ascorbate peroxidase (APX):

One week after the treatments, the enzyme activity and gene expression increase in the leaves of *smm+mdmv* plants. In the roots of *smm+mdmv* plants the gene expression and enzyme

activity of APX exceeds the values of infected plants. In the second and third week no difference can be observed in the gene expression values between *smm+mdmv* and *mdmv* plants.

The gene expression of APX increases considerably in the roots of *mms+mdmv* plants compared to infected ones. On the contrary, the enzyme activity of APX decreases in *mms+mdmv* compared to *mdmv* plants.

Changes of glutathion reductase (GR):

The gene expression of *GR* significantly increases one week after the treatments in the leaves of *smm+mdmv* plants compared to *mdmv* plants, moreover, in the third week the enzyme activity increases as well. In the root area there is a considerable increase in the gene expression value one week after the treatments in *smm+mdmv* plants, however, in the following weeks a deliberate decrease can be observed. On the contrary, three weeks after the treatments there is a significant increase in the enzyme activity of APX in the roots of *smm+mdmv* plants.

The gene expression of *GR* also significantly increases one week after the treatments in the leaves of *mms+mdmv* plants. The same week the levels of enzyme activity increase also, however, they are statistically below the values of *mdmv* plants. Two and three weeks after the treatments enzyme activity levels significantly drop in *mms+mdmv* leaves, while gene expression values exceed the values of *mdmv* plants. One week after the treatments lower enzyme activity levels can be measured in the roots of *mms+mdmv* compared to *mdmv* plants, however, the gene expression levels are higher in *mms+mdmv* than in *mdmv.* One week later there is an increase in the enzyme activity compared to *mdmv,* while the gene expression values of the two groups do not differ statistically from each other. In the third week the gene expression values of *mms+mdmv* are significantly lower than of *mdmv* plants.

Changes of guaiacol peroxidase (GPX):

Shortly after the treatments the enzyme activity of GPX decreases in *smm+mdmv* plants compared to *mdmv* ones. On the other hand the gene expression levels increase in leaves and in the first week in the roots as well.

In *mms+mdmv* plants one week after the treatments there is an increase in the values of enzyme activity, but it is still below the values of *mdmv.* On the second and third weeks the enzyme activity values further decrease, being significantly lower than the values of *mdmv.* The gene

expression levels of *smm+mdmv* leaves are significantly higher in the first and third weeks than in the *mdmv* plants, while in the second week there is only a slight increase observed. While the enzyme activity of GPX is significantly higher in *mms+mdmv* roots one week after the treatments compared to *mdmv* plants, the gene expression results are the opposite. On the second and third weeks the enzyme activity and the gene expression of *mms+mdmv* is below the values of *mdmv.*

Expressional changes of genes with specific response to MDMV-infection:

Changes of GF14-6*:*

The gene expression of *GF14-6* increases significantly in *smm+mdmv* plants. Similar increase can be seen in *mms+mdmv* plants as well, however, the increase is visible on the first and third weeks, while on the second week a decrease can be observed.

Changes of S-adenosylmethionine synthase (SAMS)*:*

The gene expression of *SAMS* in *smm+mdmv* and *mms+mdmv* plants exceeds the values of *mdmv*.

Summary:

- The chlorophyll content increases in Jubilee sweet corn due to the protective effect of SMM, which is exerted on the membranes. Moreover, the decrease in the chlorophyll content which is observed in infected Jubilee can not be seen in SMM-pretreated and infected plants.
- In contrast to *mdmv,* in *smm+mdmv* and *mms+mdmv* plants the gene expression of *GF14-6* and *SAMS* increase significantly. As a result, the intensity of the methylation pathways, taking part in plant defence mechanisms increases as well, which contributes to the production of certain defence compounds.
- As a result to the increase in the metabolic pathways, the gene expression of *cinnamic acid 4-hydroxylase* and *chalcone synthase* increases, resulting in the increase of production of secondary metabolites with defence nature.
- Following the activation of the phenylpropanoid pathway, the amount of compounds increase which emmit fluorescence at 440 and 520 nm. Furthermore, shortly after the treatments in *mms+mdmv* plants the total phenolic content increases in the leaves as well.

- As a result to the pretreatments prior to infection, shortly after the treatments the enzyme activity and gene expression of APX and GR increase significantly, therefore the plant can more effectively scavenge the reactive oxygen species.
- The activity of GPX is enhanced due to the pretreatments in infected plants, which is followed by a steady decrease in *smm+mdmv*. Based on the gene expression pattern of *GPX*, there might be a correlation between *GPX* and *GF14-6* and *SAMS.*
- The pretreatments exert a mild stress to the plants, since the ΔpH-dependent quenching and photoinhibition increase, while the number of active PS II reaction centers decrease in *smm* and *mms* plants. On the other hand, in *smm+mdmv* and *mms+mdmv* plants these effects are not observed.
- In *smm+mdmv* and *mms+mdmv* plants there is a better energy dissipation between PS I and PS II, therefore the pretreatments exert a protective effect on the photochemical systems in the case of MDMV-infection.
- Due to the mechanisms detailed above, in infected plants that were pretreated with either SMM or MMS, a significant drop in the amount of viral RNA and coat protein can be observed compared to *mdmv* plants.

Publications

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