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**MF3 (PEPTIDYL-PROLYL *CIS-TRANS* ISOMERASE
OF FKBP TYPE FROM *PSEUDOMONAS FLUORESCENS*)
– AN ELICITOR OF NON-SPECIFIC PLANT
RESISTANCE AGAINST PATHOGENS**

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Abstract

The protective capability of Microbial Factor 3 (MF3) was demonstrated for the following host–pathogen pairs: tobacco – *Tobacco mosaic virus* (TMV), tobacco – *Alternaria longipes* and white cabbage (*Brassica oleracea* var. *capitata*) – *Turnip mosaic virus* (TuMV). MF3 did not show phytotoxicity towards the plants or fungicidal effect on *A. longipes*. MF3 did not directly affect TMV but decreased viral infection in tobacco plants. MF3 concentration applied on plants was positively correlated with its protective activity. Chitosan (17 kDa) was found to enhance the protective effect of the protein and most probably served as a carrier providing MF3 transport into plant cells.

Key words: MF3, peptidyl-prolyl *cis-trans* isomerase, elicitor, plant resistance

Introduction

One of the most promising ways to search for highly effective methods of plant protection against pathogens and pests is the use of genetically incorporated plant potential. In some cases, it is possible to solve the problem of yield losses caused by phytopathogens using specific regulators influencing biochemical reactions in plant tissues.

It is well known that *Pseudomonas fluorescens* has many successful applications in plant protection since it can enhance plant growth and protect crops from diseases. The bacteria can antagonize soil-borne pathogens through various mechanisms

(e.g. bacterial siderophores inhibit plant pathogens through competition for iron, antibiotics suppress competing microorganisms, and chitinases and glucanases lyse microbial cells). Some studies have demonstrated that *P. fluorescens* can also induce systemic resistance in the plant to soil-borne fungi and plant-parasitic nematodes (Siddiqui and Shaukat 2002).

After screening *P. fluorescens* isolates from our collection, we found the bacterium strain 197 preventing multiplication of TMV in plants. Treatment of tobacco leaves with its extracts decreased the virus concentration in plants. Later, it was discovered that the active substance of the bacterial extract was a low-molecular (16.929 kDa) thermostable protein named Microbial Factor 3 (MF3). After identification of MF3-protein gene structure, it was shown that the protein had a high homology to peptidyl-prolyl *cis-trans* isomerases of FKBP type (Shumilina et al. 2005).

FK506-binding proteins (FKBPs) belong to the large family of peptidyl-prolyl *cis-trans* isomerases. Despite the fact that FKBPs are known for several decades, the cellular function of these enzymes is not yet completely understood. These proteins were demonstrated to take part in many cellular processes, such as cell signalling, protein trafficking and transcription. FKBPs associate into protein complexes, although the involvement and precise role of their foldase activity remain to be elucidated. FKBPs are involved in growth and development of plants. Disruption of genes encoding FKBPs in plants and animals has underlined the importance of the family of proteins in regulation of cell division and differentiation. The inactivation of two FKBP-like genes in *Arabidopsis* demonstrated the prominent role of the family of proteins in cell division and cell elongation in relation to two major hormones, cytokinins and brassinosteroids. An attractive hypothesis is that these two FKBP-like proteins are involved in different signalling pathways by regulating protein complex assembly or activity (Harrar et al. 2001). FKBPs are implicated in folding of newly synthesized proteins, transport and assembly of essential cellular protein complexes (Ivery 2000).

MF3 from *P. fluorescens* was the first of FKBPs which showed capability to induce plant resistance. The aim of the work was to study protective abilities of MF3 in different host plant – pathogen combinations. Direct influence of MF3 on plants and pathogens was studied. Thereupon, correlations between MF3 concentrations applied on tobacco plants and its protective activity against TMV and *Alternaria longipes* were studied. Besides, the influence of chitosan possessing elicitor activity (Kulikov et al. 2006) and enhancing transmembrane absorption of peptides (Kiling et al. 2002) on protective effect of MF3 was investigated in experiment with white cabbage plants.

Materials and methods

Tobacco plants growing

Two cultivars of tobacco were used for tests. Tobacco plants of cv. 'Xanthi' have gene N responsible for necrotic-type response to infection with TMV. Tobacco plants of cv. 'Samsun' lack the N gene. Their infection with TMV results in unlimited spread of TMV throughout plants and production of mosaic symptoms.

Tobacco (*Nicotiana tabacum*) plants were grown in a climatic chamber at 60% relative humidity and 24°C during light and dark periods (12 h each). The six-leaf-stage plants were used for experiments.

Testing the phytotoxic effect of MF3 on tobacco plants

MF3 water solution (1 mg/ml) was infiltrated into the mesophyll of fully expanded tobacco leaf using medical syringe. Physiological state (changes of colour, deformation or hypersensitive response) of treated leaves was observed each day during two weeks. Five plants were used for each experimental variant.

Testing of MF3 direct influence to TMV

Juice of TMV-infected tobacco plant was diluted with 0.01 M Na-phosphate buffer.

The diluted solution was centrifuged at 15 000 g for 20 min. Supernatant was transferred into two tubes and solution of MF3 was added to the first tube up to final concentration in solution 10 µg/ml. An equal volume of distilled water was added into the second tube. After 24 h of incubation TMV was isolated from solutions by ultracentrifugation at 105 000 g for 90 min. TMV-contained pellet was dissolved in water and centrifuged at 15 000 g for 20 min to remove insoluble material. Supernatant containing purified TMV was used for inoculation of tobacco leaves (cv. 'Xanthi' NN.) The number of lesions was counted up for three days after inoculation.

In the test and all experiments described below, inoculation with TMV was carried out by rubbing with carborundum.

Testing of MF3-induced resistance of tobacco plant to TMV

Five tobacco plants (cv. 'Samsun' nn) were used for each treatment. All leaves of plants (with the exception of the bottommost leaves) were sprayed with MF3 water solutions (concentrations: 10, 50 and 100 µg/ml) or water in control variant (~1 ml per each plant). Next day, the bottommost leaf of each plant was inoculated with TMV. For this purpose, 10⁴ diluted juice of TMV-infected tobacco plant was used.

TMV presence was tested in all plants with ELISA during 80 days after the inoculation. The ELISA test was carried out using a TMV ELISA kit (All-Russian Potato

Research Institute, Korenevo, Moscow region) according to the standard assay procedures described by the manufacturers.

Testing of MF3-induced resistance of necrotic-type response tobacco plants to TMV

Five detached tobacco leaves (cv. 'Xanthi' NN) were used for each treatment. One half of each leaf was treated with MF3 water solution in concentrations 0.1, 1, 10, 20, 30 and 50 µg/ml, the other one was treated with water. In this experiment, rubbing with carborundum was used not only for inoculation but also for leaf treatment with MF3 to allow the protein penetration into plant cells. Leaves after treatment were placed in a moist chamber at 22°C. Next day, both halves of the leaves were inoculated with TMV, as described previously. Number of lesions was counted up in three days for each leaf half separately.

Testing the duration of tobacco leaf resistance to TMV

Tobacco plants (cv. 'Xanthi' NN) were used for the test. One half of each leaf was treated with MF3 water solution (concentration 1 µg/ml), the other one was treated with water. Treatment of leaves with the protein was carried out by rubbing with carborundum. After 1, 2, 3, ..., 21 days both halves of treated leaves were inoculated with TMV. After inoculation, leaves were placed in a moist chamber at 22°C. Number of lesions was counted up in three days for each leaf half separately.

Studying the direct influence of MF3 on *Alternaria longipes* spore germination

Droplets (100 µl) of a mixture of MF3 in different concentrations (0.7, 7 and 70 µg/ml) and *A. longipes* spore suspension (3×10^4 spores per 1 ml) in 50 mM glucose were placed on object-plate and incubated in a moist chamber at 20°C overnight. Germination of spores in 50 mM glucose was used as reference. The number of germinated spores was counted in treatments and under microscope.

Test of tobacco plant resistance to *Alternaria longipes*

Five detached tobacco leaves (cv. 'Xanthi' NN) were used for each experiment. Leaves were treated with MF3 water solution (concentrations 0.7, 7 and 70 µg/ml). The next day, undersides of the same leaves were inoculated with *A. longipes* spore suspension (5×10^4 spores per 1 ml) in 50 mM glucose and Tween 20 (0.20%). Inoculated leaves were maintained at 20°C in moist chamber. Number of lesions was scored in seven days after inoculation.

Chitosan preparations

Low-molecular chitosan preparations with average viscosimetric weight 17 kDa was supplied by Dr. V.P. Varlamov (Centre “Bioengineering” of Russian Academy of Sciences, Moscow). The preparations were obtained from chitosan of Kamchatsky crab shell chitin with a degree of deacetylation of 85% and molecular weight 700 kDa (product of “Bioprogress”, Russia). To produce 17-kDa chitosan, the high-molecular chitosan preparations were subjected to enzymatic hydrolysis using industrial preparation Celloviridine G20x (Russia) that contained glucanase, xylanase and cellulase from *Trichoderma reeseii*.

Study of white cabbage resistance to *Turnip mosaic virus* (TuMV)

White cabbage (*Brassica oleracea* var. *capitata*, cv. ‘Krautman’) plants were grown in a climatic chamber at 70% relative humidity and 24°C during light and dark periods (12 h each). Five of the fourth-leaf-stage plants were used for the experiments.

Test was carried out as described above for tobacco plants (cv. ‘Samsun’ nn) and TMV. Plants were treated with water (control), MF3 water solution (10 µg/ml), chitosan solution (3 µg/ml) or mixture of MF3 and chitosan (in concentrations 10 and 3 µg/ml, respectively). TuMV was tested in all plants using standard ELISA till 23 days after the inoculation.

Statistical analysis

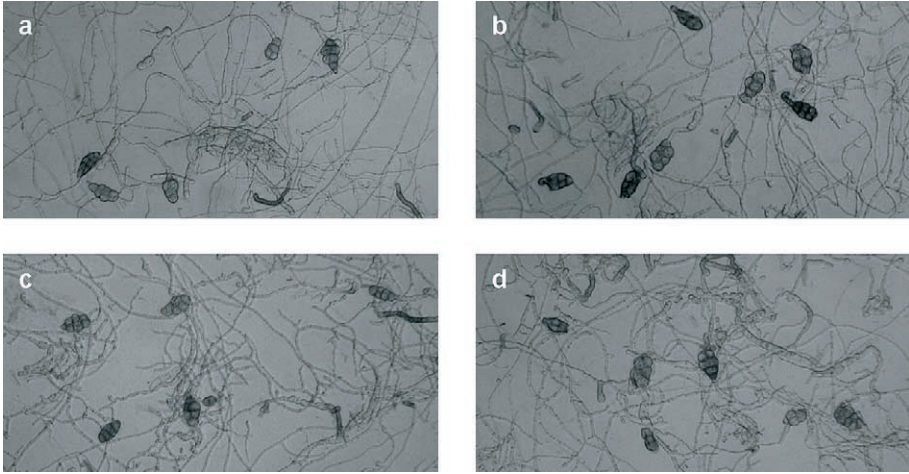
All tests were carried out at least three times. Student’s t-test was used for statistical analysis of results ($P < 0.10$).

Results

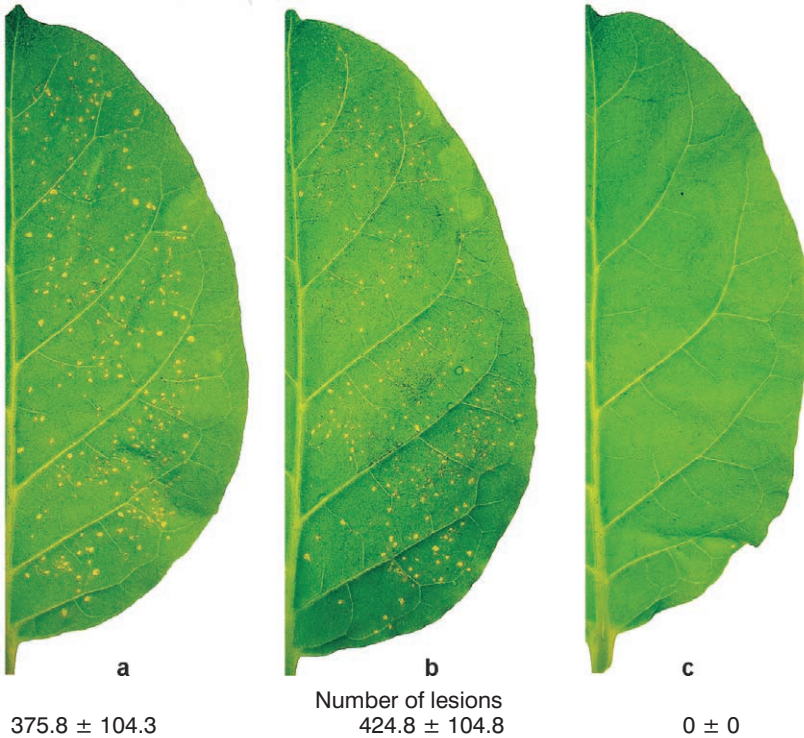
Infiltration of MF3 water solution in concentration 1 mg/ml into tobacco leaves did not cause changes in their physiological state (discolouration, deformation or hypersensitive response). Leaves treated with MF3 looked as control leaves during the entire test time (two weeks).

To ensure that protective effect of MF3 was related to induction of plant resistance rather than fungitoxic impact on the fungus, the different concentrations of the protein were mixed with *A. longipes* spore suspension and incubated overnight. No difference in the germination and hyphae thickness between MF3 treated spores and control ones was found (Phot. 1).

Incubation of viral preparations with MF3 did not influence ability of TMV to infect tobacco plants. The numbers of lesions on leaves infected by both TMV incubated in water and TMV preincubated in MF3 were equal. At the same time, no lesions were observed on leaves treated with MF3 and subsequently inoculated with TMV (Phot. 2).



Phot. 1. Influence of MF3 in different concentrations on *Alternaria longipes* spores germination: a – water (control), b – MF3 – 0.7 µg/ml, c – MF3 – 7 µg/ml, d – MF3 – 70 µg/ml (photo by D. Shumilina)



Phot. 2. Lesion development on tobacco leaves inoculated with: a – TMV, b – TMV previously incubated with MF3; c – the half of tobacco leaf treated with MF3 and inoculated with TMV (photo by D. Shumilina)

Tobacco plants of cv. 'Samsun' nn pretreated with MF3 water solution in concentrations of 10, 50 and 100 $\mu\text{g}/\text{ml}$ did not contain TMV amount sufficient for detection by ELISA during 50 days. At the same time, control plants showed high level of the virus content in juice already in the second week after inoculation.

After 60 days of experiment some plants treated with low concentration of MF3 were shown to contain TMV in juice, but in significantly lower amount than in control plants (Fig. 1).

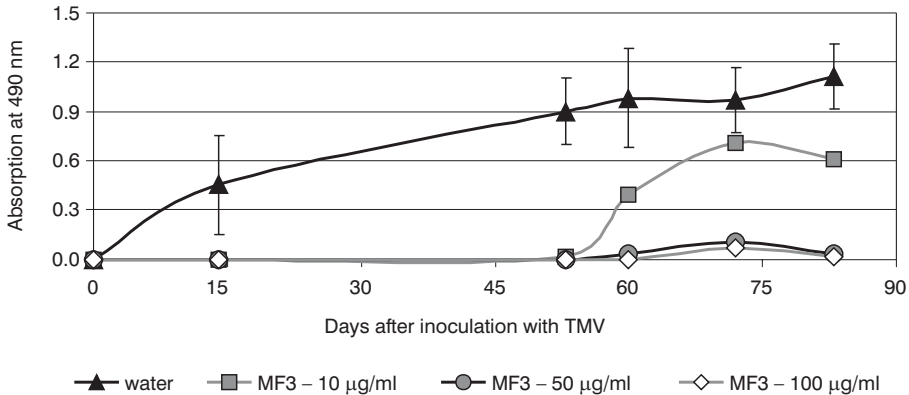


Fig. 1. Influence of tobacco (cv. 'Samsun' nn) plants pretreatment with MF3 water solution on TMV development

Treatment of one half of tobacco leaf (cv. 'Xanthi' NN) with MF3 and the neighbouring one with water is suitable for study of MF3 ability to induce trans-laminar resistance. It was shown that the number of lesions developed after infection with TMV on the leaf halves treated with MF3 water solution (concentrations 0.1–10 $\mu\text{g}/\text{ml}$) was significantly smaller in comparison with other parts of leaves or with the untreated control leaves. Treatment of one half of leaf with MF3 in concentration more than 10 $\mu\text{g}/\text{ml}$ before inoculation of the whole of leaf with TMV did not induce lesion development on these halves and reduced lesion number on neighbouring halves in comparison with control leaves (Fig. 2).

Tobacco leaves (cv. 'Xanthi' NN) treated with 1 $\mu\text{g}/\text{ml}$ MF3 water solution were found resistant to TMV for three weeks after the treatment. In the same time lesions developed in great number on leaf halves treated with water (Fig. 3).

Development of *A. longipes* on tobacco leaves was observed for seven days after inoculation. Necroses developed numerously on leaves treated with water and MF3 water solution in concentration 0.7 $\mu\text{g}/\text{ml}$. On leaves pretreated with MF3 in concentrations of 7 and 70 $\mu\text{g}/\text{ml}$ development of the symptoms was significantly lower (Fig. 4).

Spraying tobacco plants with MF3 induced resistance to TMV and *A. longipes*. At the same time, similar treatments of white cabbage plants did not induce any increase of resistance to TuMV.

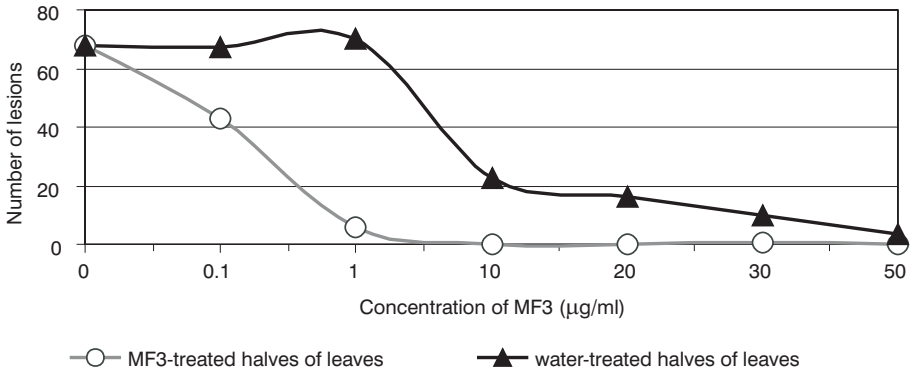


Fig. 2. Influence of tobacco (cv. 'Xanthi' NN) leaf halves pretreatment with MF3 water solution on lesion development after TMV inoculation

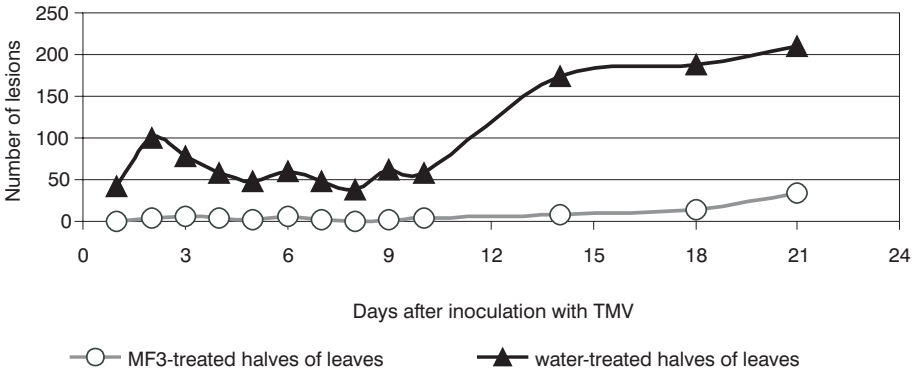


Fig. 3. Influence of tobacco (cv. 'Xanthi' NN) leaf halves pretreatment with MF3 water solution on development of TMV lesions for three weeks

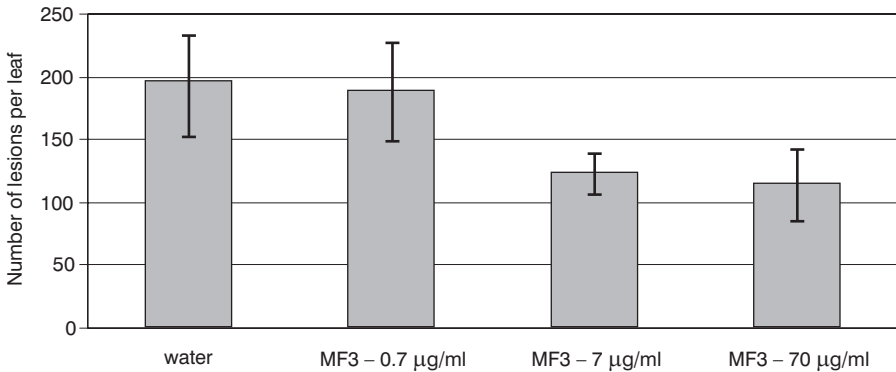


Fig. 4. Influence of tobacco (cv. 'Xanthi' NN) leaves pretreatment with MF3 water solution on development of *Alternaria longipes*

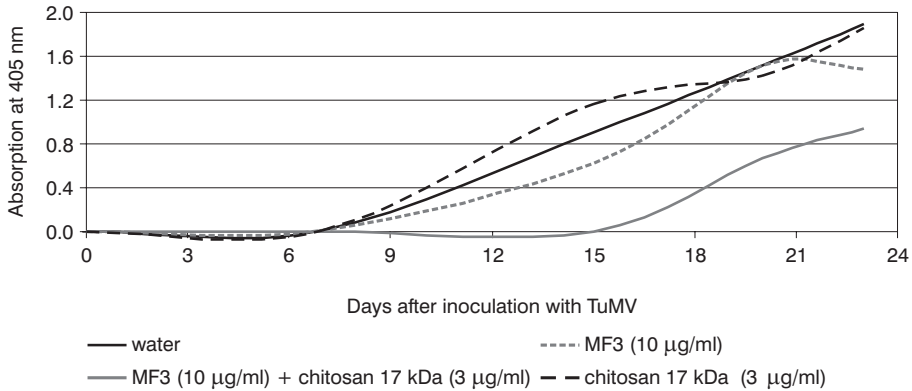


Fig. 5. Influence of white cabbage (cv. 'Krautman') plants pretreatment with MF3, chitosan or mixture of both substances on TuMV concentration

Treatment of the white cabbage plants with water, chitosan solution and water solution of pure MF3 did not induce the increase of resistance to TuMV. Virus concentration in the plant juice was high already on the 15th day after inoculation. Plants treated with mixture of MF3 and chitosan were more resistant to TuMV. Virus concentration increase in these plants was slower than in control plants, for at least seven days (Fig. 5).

Discussion

Our study of MF3 from *P. fluorescens* showed that some peptidyl-prolyl *cis-trans* isomerases of FKBP type can operate as inducers of disease resistance. Treatment of 'Xanthi' plants with N gene encoding necrotic-type response to TMV infection resulted in increasing the intensity of defence reaction. In some cases, the response was so quick that no lesions developed on MF3-treated leaves, and they look as healthy control (uninoculated) leaves. Providing lesions were visible, their number on the protein-treated leaves was always lower than on leaves untreated with MF3. The treated leaves became more resistant after only one application of the protein. Such a quick and intensive response can be important for the restriction of TMV spread in plants. Usually the size of lesions decreased on MF3-treated, suggesting modification of response type under the influence of protein.

In tobacco leaves (cv. 'Xanthi' NN) treated with MF3 in concentrations higher than 10 µg/ml, trans-laminar resistance was induced. It prevented lesion development on tested halves and reduced the number of lesions on neighbour halves as compared to control leaves.

MF3 was shown to influence TMV through plant, though TMV did not lose activity and induced lesions development on tobacco leaves after 24-h incubation in the protein.

For the study of MF3 systemic influence on cv. 'Samsun' nn plants susceptible to TMV were used. The virus was detected in juice of untreated plants after two weeks, while MF3-treated 'Samsun' plants were more resistant to TMV. The virus in those was not detected by ELISA for 50 days after inoculation. TMV accumulated neither in treated leaves nor in the new grown ones. Apparently, MF3 was capable of inducing systemic resistance in the tobacco plants against TMV.

Ability of MF3 to protect plants did not concern only the viral pathogen. The protein was shown to be also an inducer of tobacco resistance to *A. longipes*. MF3 did not influence *A. longipes* spore germination, hyphae thickness or length. That suggests that MF3 have no direct negative effect on the fungus. Presumably, MF3 contacts with specific receptors of plant cells and induces defensive reactions that are common for protection of plants against viral and fungal pathogens. It was known that fungal signals were perceived by plasma membranes of plants (Shiraishi et al. 2001). New findings showed that plants were able to recognize and respond rapidly to elicitors and suppressors even on the surface of uninjured tissues (Shiraishi et al. 2001). Cell wall, the most exterior and plant-specific organelle barrier may play a crucial role in determining plant host – parasite specificity, at least in species–species combinations (Shiraishi et al. 2001). These results are in agreement with our results when treatment by spraying with MF3 water suspension was sufficient for protection of plants against viral and fungal pathogens.

The use of protein elicitors in agricultural practice is impossible without formulation. It is quite possible that morphological features of tested plants influence the MF3 efficacy in plant resistance induction. Leaves of tobacco plants are pubescent and have no wax coating as compared to white cabbage plants. A proposition arose that MF3 needed a carrier for transfer into cabbage cells. We took the first step for formulation of a preparation based on MF3.

Chitosan was chosen as a possible MF3 carrier to plant receptors on the basis of its properties to bind proteins and penetrate into plant cells. Chitosan is able to elicit plant resistance to some pathogens itself, and we expected a synergic effect of MF3 and low molecular chitosan mixture. Test on white cabbage inoculated with TuMV with the use of such a mixture gave positive results and suggested that combination of MF3 with chitosan can be used in formulation of biopreparation based on MF3.

Thus, MF3 is promising as an elicitor for induction of non-specific plant resistance against pathogens and development of new biopreparations.

Streszczenie

MF3 (PEPTYDYLO-PROLYLO *CIS-TRANS* IZOMERAZA TYPU FKBP
Z *PSEUDOMONAS FLUORESCENS*) JAKO ELICYTOR NIESPECYFICZNEJ
ODPORNOŚCI ROŚLIN NA PATOGENY

Mikrobiologiczny czynnik MF3 (ang. *microbial factor 3*), białko *Pseudomonas fluorescens* o aktywności enzymatycznej, wykazał zdolności ochronne w następujących układach roślina-gospodarz – patogen: tytoń – wirus mozaiki tytoniu

(TMV), tytoń – *Alternaria longipes* i kapusta biała (*Brassica oleracea* var. *capitata*) – wirus mozaiki rzepy (TuMV). MF3 nie był toksyczny dla roślin. Nie miał też działania fungicydalnego na *A. longipes* ani nie oddziaływał bezpośrednio na TMV, lecz ograniczał nasilenie choroby wirusowej w roślinach tytoniu. Wystąpiła pozytywna korelacja pomiędzy stężeniem zastosowanego MF3 a jego aktywnością ochronną. Chitozan (17 kDa) zwiększał efekt ochronny tego białka, najprawdopodobniej ułatwiając transport MF3 do komórek rośliny.

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