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SEARCH FOR THE ACTIVE CENTER OF PEPTIDYL-PROLYL cys/trans ISOMERASE FROM *Pseudomonas fluorescens* RESPONSIBLE FOR THE INDUCTION OF TOBACCO (*Nicotiana tabacum* L.) PLANT RESISTANCE TO TOBACCO MOSAIC VIRUS

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Abstract

The induction of pathogen resistance with biogenic elicitors is now considered as a promising plant protection method. Elicitors obtained from fungal, bacterial, and oomycetic pathogens can be of peptide, glicoproteid, lipid, and oligosaccharide origin. The first reported protein elicitor was harpin isolated from *Erwinia amylovora*. Based on this elicitor, a Messenger® preparation intended to protect plants from a wide range of pathogens was developed and commercialized in USA. The MF3 protein able to induce the systemic resistance of plants to viral and fungal pathogens was isolated from the strain 197 of Pseudomonas fluorescens in the course of our earlier studies. Such a wide range of action assumed prospectivity of MF3 using as a potential plant protection remedy. The full amino acid sequence of this protein was determined, and its high homology to the FKBP-type peptidyl-prolyl cys/trans isomerases of the same bacteria was demonstrated, so the isolated protein was called Pf197_PPIase. As a rule, active centers of the majority of known elicitor proteins are localized at the most conserved domains. We supposed that the active center of Pf197_PPIase responsible for its ability to induce the resistance is localized within the most conserved sequence of this protein. The calculation of this sequence using a PROSITE bioinformational resource showed it contains sites composed of arginine and lysine and subjected to the tripsinolysis. The preparations of Pf197_PPIase were obtained from the recombinant strain of *Escherichia coli* BL21(DE3)+plMF3 — the superproducer of Pf197_PPIase. The treatment of Pf197_PPIase with tripsin resulted in the loss of its elicitor properties that indirectly confirmed our hypothesis about the responsibility of the studied conserved domain for the elicitor activity of the MF3 protein. A Pf_29ac oligomer consisting of 29 amino acids and corresponding to the revealed conserved region was obtained by a chemical synthesis (Pushchino affiliated Branch of the Institute of Bioorganic Chemistry). The further experiments showed that equimolar concentrations of Pf197_PPIase and Pf_29ac induced a similar level of resistance of tobacco plants to Tobacco Mosaic Virus (TMV). These results were confirmed in biotests when necroses were calculated on the surfaces of the inoculated tobacco leaves. The leaves were treated with preparations and incubated in wet chambers for 24 hours at 22 ºC. After that the leaves were inoculated with viral suspension and incubated in wet chambers during 3-4 days at 22 ºC. The obtained results permitted us to conclude the conserved domain of Pf197_PPIase alone is sufficient for the induction of the TMV resistance in tobacco. The further determination of a minimum size of the active center able to provide the elicitor activity is planned.

Keywords: induced disease resistance of plants, protein elicitors, tobacco mosaic virus, peptidyl-prolyl cis/trans isomerases, conserved protein domains

Agrotechnologies based on application of biological substances are a promising to-date approach in modern agricultural science [1-3]. These technologies are also used for plant protection against diseases including biopesticide application and the induction of pathogen resistance by biogenic elicitors [4-7]. Due to searching biologically active substances the microbial proteins capable of eliciting resistance in plants to various pathogens and pests were identi-

fied [8-11]. One of these proteins, called MF3 (microbial factor 3), was isolated by us from the *Pseudomonas fluorescens* Pf197 strain [12]. Studies have shown that MF3 had no direct effect on the phytopathogens, but could increase plant resistance to a number of different viruses and fungi, in particular, promote the tobacco systemic resistance to tobacco mosaic virus (TMV). Such a wide range of activity pointed to prospects in using MF3 as a potential remedy for crops. Complete amino acid sequencing [13] showed a high homology of MF3 to peptidyl-prolyl cis/trans isomerase (PPI-ase) of FKBP-type of the same bacteria [14], so MF3 was called Pf197_PPI-ase. In contrast to other proteins inducing plant resistance to pathogens, particularly harpins [15], Pf197_PPI-asa has a relatively low molecular weight of 16.9 kD [13], and high thermal stability, which facilitates its isolation and purification from cell lysates. Natural degradation poses certain restrictions on the proteins used as plant protection agents. However, it was shown that in many protein inducers of disease resistance in plants not only native molecule, but a part thereof had an elicitor activity. Thus, peptide flg22, encompassing the most conservative amino acid sequence of eliciting bacterial flagellin, can induce, similarly to native flagellin, an oxidative burst in *Arabidopsis*, the PR-protein expression and ethylene synthesis, and also stimulate the callose deposition in plant tissues [16]. This 22-amino-acid peptide is responsible for the elicitor properties, flagellin binding and the recognition by plant cells [17]. It is also known that Arabidopsis plants specifically recognize N-terminal domain of another eliciting protein, the bacterial elongation factor Tu (EF-Tu), which leads to activation of the plant protective response system. A similar effect can be achieved by using inducer peptide elf18, which is a 18 amino acid long fragment of EF-Tu [18]. Cold shock protein from *Micrococcus lysodeikticus* serves as a non-specific elicitor of defense response in *Solanaceae*. The eliciting activity of this protein is due to csp15, the 15-amino-acid peptide corresponding to its conserved domain [19, 20].

When studying Pf197_PPI-ase, we firstly discovered a new property of peptidyl-prolyl cis/trans isomerases of FKBP-type, namely the ability to induce plant resistance to pathogens, in particular, to tobacco mosaic virus (TMV). However, the mechanism of this phenomenon remained unknown. In particular, there was no information about whether this protein comprised the amino acid sequence responsible for such activity. In this paper, we firstly identified the polypeptide fragment of the PPI-asa from *P. fluorescens* 179, responsible for the elicitor properties of this protein, and obtained the experimental evidence of its role in the expression of the tobacco plant tolerance to TMV.

The aim of submitted work was to determine the polypeptide fragment of PPI-asa from *P. fluorescens* 179 responsible for the induction of pathogen-resistance in various plants.

Technique. Pf197_PPI-ase was isolated from *Escherichia coli* BL21 (DE3)+plMF3 strain, overproducing PPI-ase, which was grown on YT medium (100 ml) in flasks; ampicillin was added to 100 μg/ml immediately before inoculation. The stock culture of BL21 (DE3)+plF3 stored at –20 °C in 25 % glycerol was used for inoculation. Flasks were incubated on a thermo-shaker Excella™ E-25/25R (New Brunswick Scientific Co., Inc., USA) at 250 rev/min (5 cm eccentricity) and 37 °C for 20-22 hours. The resulting culture was centrifuged for 30 minutes at 4000 g. The precipitated cells were suspended in buffer (50 mM Tris-HCl, 0.15 M NaCl, 2 mM EDTA, pH 8.0) containing lysozyme (2 mg ml) and incubated for 30 min at 37 °C. The cell suspension was sonicated (5 times for 40 sec) and placed in a boiling water bath (20 min, 100 °C) with occasional stirring, then rapidly ice-cooled to 0 °C and centrifuged (4000 g, 30 min).

The supernatant, containing approximately 200 mg Pf197_PPI-ase per

liter was successively ultra-filtered on an Amicon 8050 cell (Millipore Corp., USA). The supernatant was separated using a selective membrane MWCO 100 kDa, and then the obtained permeate was filtered through the membrane MWCO 10 kDa. The ultrafiltration enabled the target protein concentration and separation from high and low molecular weight components of the supernatant.

The clarified cell lysate was applied to a column (25×50 mm) with Chelating Sepharose ($Ni2^+$) (Pharmacia, Sweden) and 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M NaCl. The column was rinsed thoroughly with 50 mM Tris-HCl buffer (pH 7.5), containing 1 M NaCl. Protein was eluted with a linear imidazole gradient at 3 ml/min flow rate and 300 ml gradient volume. The eluate (50-60 ml) was dialyzed against 2 liters of distilled water at 4 °C. Since PPI-asa precipitation at an acidic pH, water was alkalinized with NaOH to pH 7.0. The purity of the resulting PPI-ase solution was assessed by gel electrophoresis according to Laemmli [21]. For long-term storage Pf197_PPI-ase was lyophilized and stored hermetically in tubes at –20 °C.

To identify the most conserved regions, the amino acid analysis of the previously found protein sequence [12] was performed using bioinformatics resource PROSITE [22, 23].

To confirm the role of the conserved region in the tolerance induction, the Pf197_PPI-ase was trypsinized after heat pre-denaturation at 95 °C for 15-20 min in 50 mM Tris-HCl buffer, pH 8.0, with 8 M urea and 4 mM mercaptoethanol. After cooling the solution was diluted with 50 mM Tris-HCl buffer containing 1 mM CaCl2 (pH 7.6) to 1 M urea. Trypsin (1:20 w/w) was added to the heat denatured protein. After 1 hour incubation at 37 °C the reaction was stopped by 1 mM PMSF. Completeness of the hydrolysis was monitored by the SDS-polyacrylamide gel electrophoresis.

The 29-amino-acid oligopeptide corresponding to a fragment of one of the Pf197_PPI-ase conserved sequences (hereinafter referred to as Pf_29ak), was synthesized in the Pushchino Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences.

Protective activity of tested agents was evaluated in a bioassay using tobacco (*Nicotiana tabacum* L.) cultivar Xanthi (NN) grown in a climate chamber at 16 hour photoperiod and 24 °C/20 °C (day/night). At 3-4 true leaves, the leaves of the same tier were separated. On one half of each leaf we applied aqueous or buffer solutions containing equimolar amounts of native Pf197_PPI-ase, or the resulting peptides after its tryptic cleavage, or the synthesized Pf_29ak oligopeptide. The other (control) half of the same leaf was treated with distilled water or appropriate buffer solutions, containing all the components used on an adjacent half except the protein or peptides tested. Thus, when tryptic peptide activity was assessed, it was the proteolysis buffer (see above) with inactivated trypsin, and for Pf_29ak test it was 0.1 % bovine serum albumin (BSA, aqueous solution) used to dissolve the oligopeptide. In estimation of TMV infectivity, the leaves with both halves treated by distilled water before inoculation were an additional control. A total of 5 leaves per Pf197_PPI-ase, tryptic peptides or Pf_29ak oligopeptide were incubated for 1 day at 22 °C in a wet chamber, and then inoculated with juice of tobacco plants pre-infected with TMV, with carborundum added. The juice was diluted by distilled water so that a 60-μl aliquot evolved 100 to 200 necroses in control. The inoculated leaves were kept in a wet chamber at 22 °C, and after 3-4 days of incubation the necroses were counted. Assessments were replicated at least three times per each candidate agent.

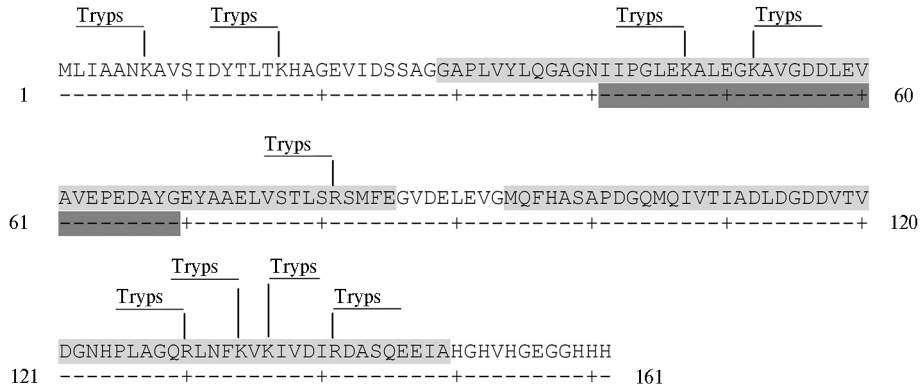
STATISTICA 6.0 (SoftStat, Inc., USA) analytics software was used for data processing, with the average standard deviation and standard error of the

arithmetic mean determined. The differences between the variants are significant at $p \geq 0.05$ according to the *t*-test, whenever it is not specifically stated.

Results. Since Pf197_PPI-ase can protect phylogenetically distant plants against phylogenetically unrelated pathogens [12], it obviously induces a non-specific resistance, and the protein itself may activate protective responses that are common to different plant species [24]. Elicitors of Mamre/PAMP-type (Microbial Associated Molecular Pattern/Pathogen Associated Molecular Pattern) which are microbial metabolites, including proteins essential for microorganism and therefore always synthetized in microbial cells, are known as typical inducers of protectiv responses [25, 26]. The recognition sites for interaction with the plants are usually the most conservative sequences of eliciting molecules, such as peptide fragments flg22, elf18 and csp15 [9, 27]. Based on this, we hypothesized that Pf197_PPI-asa may also comprise a peptide fragment associated with its activity against plant pathogens, in particular TMV, and it is likely that this fragment is localized in the conservative region of the molecule.

Due to ScanProsite program [22, 23] used for searching genes homologous to a gene encoding Pf197_PPI-ase, more than 45 similar PPI-ase genes were identified in different organisms [28]. Furthermore, two major conserved regions of Pf197_PPI-ase molecule were found (Fig.). One of them embodies the most conservative 29-amino-acid sequence.

Using PeptideCutter program (http://ca.expasy.org/tools/peptidecutter/) we have chosen trypsin as a specific enzyme (see Fig.) to verify the possible eliciting activity associated to this sequence. Loss or reduction activity in cleaved Pf197_PPI-ase could show that the sequence we found within the domain is associated to the protective function. The trypsin treatment should produce 10 fragments of target protein including those resulting from two internal trypsin cleavage sites in 29-amino-acid sequence (see Fig.).



Trypsin cleavage sites (Tryps) **in Pf197_PPI-asa of FKBP-type from *Pseudomonas fluorescens*.** Conservative sequences are light gray marked, the most conservative sequence (Pf_29ak) is dark gray marked.

As a test system we used the TMV—Xanthy tobacco cultivar model. It is convenient, as enables enough accurate quantification of induced tolerance by counting necroses.

Our experiments showed that a pre-treatment with resulting peptides, unlike native Pf197_PPI-ase, did not prevent TMV necrosis, that is, the tryptic cleavage deprived the Pf197_PPI-ase of its ability to induce tolerance. On the Pf197_PPI-ase-treated leaf halves the necrosis number was significantly less compared to that for tryptic peptides (Table 1). Thus, Pf197_PPI-ase treatment with trypsin resulted in inactive peptides, indicating that the PPI-ase region re-

sponsible for elicitor properties was damaged.

1. Bioassay of TMV infection in tobacco (*Nicotiana tabacum* L.) cultlivar Xanthy leaves pre-treated with Pf197_PPI-ase and tryptic peptides ($X \pm x$)

Variant	Tested solution	Necroses per leaf half
I	Tryptic peptides, 1 μg/ml	93.4±17.0[a]
	Control	96.2±18.1[a]
II	Tryptic peptides, 1 μg/ml	116.2±20.4[b]
	Control + Pf197_PPI-ase, 1 μg/ml	12.4±1.8[b]

N o t e. Tryptic peptides were applied on one half of each leaf, and the other half served as control. In control, trypsin was added to proteolysis buffer. The same letters mean no statistical differences between the data ($p > 0.05$).

One of the most conservative sequences in the Pf197_PPI-ase molecule includes a 29-amino-acid peptide IIPGLEKALE GKAVGDDLEV AVEPEDAYG (Pf_29ak). We examined whether the Pf_29ak is capable of inducing resistance to TMV in tobacco leaves in a climate chamber. To minimize the proteinase impact, 0.1 % BSA was added to the peptide solution. It was shown (Table 2) that the pre-treatment with three Pf_29ak concentrations induced resistance to TMV both in treated and (to some extent) in adjacent untreated leaf haves, i.e. translaminar effect occurred. Thus, the synthetic oligopeptide was found to be capable of inducing resistance to TMV in tobacco leaves.

2. Tobacco mosaic virus infection in tobacco (*Nicotiana tabacum* L.) cultivar Xanthy leaves pre-treated with Pf_29ak and Pf197_PPI-ase ($X \pm x$)

Treatment	Leaf half	Necroses per leaf half	Protection, %
BSA (0.1 %)	Control	294.9±13.2	
Pf_29ak (0.5 nM) + BSA (0.1 %)	Test	96.7±44.7	67.2
	Control	235.0±25.5	20.3
Pf197_PPI-asa (5.9 nM) + BSA (0.1 %)	Test	193.2±9.7	34.5
	Control	251.7±41.5	16.7
Pf_29ak (5 nM) + BSA (0.1 %)	Test	62.3±22.0	78.9
	Control	186.3±58.5	36.8
Pf197_PPI-asa (59 nM) + BSA (0.1 %)	Test	49.7±17.3	83.7
	Control	277.5±33.1	5.9
Pf_29ak (50 nM) + BSA (0.1 %)	Test	60.8±15.3	79.4
	Control	200.2±75.7	32.1

N o t e. BSA — bovine serum albumin.

We compared the peptide and the parent protein by expressing their amounts in nanomoles. Thus, when using Pf197_PPI-ase at 1 μg/ml, corresponding to 59 nM, and at 5.9 nM, the protective effect was 80-90 % and about 30 %, respectively. Meanwhile, 5 nM Pf_29ak resulted in 78.9 % protective effect. Thus, the synthetic peptide had no less protective properties than the native Pf197_PPI-ase molecule.

FK506-binding proteins (FKBP) belong to a large family of peptidyl-prolyl cis/trans isomerases [28]. Though these proteins are known for a long time, their intracellular function is not fully understood. FKBPs are involved in many essential intracellular processes such as signaling, traffic and transcription. Their role in plant growth and development has been shown in experiments with blocking plant genes encoding FKBP-type proteins. Inactivation of these two genes in Arabidopsis showed the involvement of these proteins in the regulation of cytokinins and brassinosteroids biosynthesis. Due to different signaling pathways these two FKBP-type proteins participate in the regulation of complex protein assembly or activity [29]. FKBP-type proteins are involved in packaging newly synthesized polypeptides, as well as in transport and assembly of cellular protein complexes [30]. Additionally to the PPI-ase capability of growth inhibition in another organism due to competitive binding receptors, some cyclophilins may directly inhibit fungi. Thus, 20 kDa cyclophilin C-CyP from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) is fungitoxic and inhib-

its the in vitro growth of *Candida albicans*, *Rhizoctonia solani*, *Botrytis cinerea*, *Trichoderma harzianum* and *T. viride*. The development of *Fusarium solani* and *F. oxysporum* mycelia was influenced to a lesser extent, and, finally, C-CyP had no effect on *Aspergillus flavus* [31]. Unlike this protein, the Pf197_PPI-ase we isolated from *P. fluorescens*, shows no direct antifungal or antiviral action, however, it is the first PPI-ase of FKBP-type for which the ability to induce plant resistance to pathogens has been proved.

In order to find out whether Pf_29ak determines the minimum size of the active center, we plan to build a library of shorter oligopeptides within this sequence. Testing these peptides will reveal the minimum portion of the Pf197_PPI-ase molecule sufficient to induce plant resistance to pathogens, or prove that the whole Pf_29ak sequence is required.

Thus, the Pf197_PPI-ase amino acid sequence comprises a portion, damage of which leads to loss of inducing activity. This fragment corresponds to the most conservative region and consists of 29 amino acids. Synthetized hypothetical oligopeptide Pf_29ak and the native Pf197_PPI-ase molecule have the same elicitor activity in equimolar concentrations. In other words, the active site of the Pf197_PPI-ase responsible for its ability to induce plant resistance to pathogens consists of at least 29 amino acids.

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