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Enhancing resistance of transgenic carrot to fungal pathogens by the expression of *Pseudomonas fluorescence* microbial factor 3 (MF3) gene

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Abstract

Microbial factor 3 (MF3) gene from a plant-growth promoting rhizobacteria *Pseudomonas fluorescence* was introduced into carrot genome using *Agrobacterium*-mediated transformation. The obtained transgenic plants expressing MF3 protein were grown in a glasshouse and evaluated for their resistance to phytopathogens. The assays were performed by the assessment of disease symptoms developing on the detached leaflets and petioles inoculated by three fungal pathogens. The results showed that transgenic plants had significantly enhanced resistance to *Alternaria dauci*, *Alternaria radicina* and *Botrytis cinerea*, on average, by 20–40% in comparison to the non-transformed control plants. This is the first report of enhancing plant resistance by expressing the bacterial protein homologous to the family of FK506-binding proteins.

Keywords: *Agrobacterium tumefaciens*; Binding proteins; *Daucus carota*; FKBP; Genetic transformation; Phytopathogens; Plant resistance

1. Introduction

Plant resistance to phytopathogens is determined by numerous mechanisms operating during plant–microbe interactions. Plants are defended from pathogen invasion by the development of morphological and structural barriers, and by the production of antimicrobial compounds [1]. In spite of the constitutive protection, plants developed defense mechanisms, which are induced after the pathogen attack. The inducible resistance operates first locally leading to a spatially restricted tissue necrosis known as the hypersensitive reaction. Additionally, the recognition of the pathogen intrusion can elicit induced systemic resistance (ISR), a non-specific resistance in distal parts of the host [2,3]. ISR develops due to a signal dispersion mediated by the messenger molecules like ethylene, jasmonic acid, salicylic acid or nitric oxide. These plant-signaling molecules bind to specific proteins involved in transcription of pathogenesis-related genes [4].

Despite their own defense mechanisms, plants can be protected from pathogens by antagonistic microorganisms colonizing rhizosphere. *Pseudomonas fluorescence* are soil-borne plant-growth promoting rhizobacteria (PGPR) known also as effective suppressors of several plant diseases, reviewed by Hass and Défago [5]. The bacteria produce antibiotics directly affecting phytopathogens, but are also capable of inducing systemic resistance in plants challenged to pathogenic fungi as well as nematodes [6,7].

Recently, screening of several PGPR resulted in the identification of *P. fluorescence* strain PM197 enhancing plant resistance. The bacterial extract applied to tobacco plants induced their resistance to tobacco mosaic virus (TMV). The active component of the extract was a novel protein, a thermostable, low molecular mass (16.9 kDa) microbial factor 3 (MF3) [8]. The resistance assays with several plant–pathogen pairs showed that pure MF3 protein stimulated plant resistance. MF3-treated tobacco leaves inoculated with TMV had reduced number of

Abbreviations: FKBP, FK506-binding proteins; ISR, induced systemic resistance; MF3, microbial factor 3; PGPR, plant-growth promoting rhizobacteria; RAUDPC, relative area under the disease progress curve.

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disease lesions by 71% in comparison to the control. Similar effect (57%) was observed on wheat leaves inoculated with *Septoria nodorum*. In another experiment, the development of disease symptoms caused by TMV on tobacco leaves pre-treated with water solution of MF3 was delayed by about 8 weeks and the plants exhibited less severe symptoms than the untreated control. The protective effect of MF3 on white cabbage after inoculation of leaves with turnip mosaic virus (TuMV) was not observed, however, the disease development was delayed by about 1 week. It was also demonstrated that MF3 elicited ISR. The pre-treatment only of a one half of a tobacco leaf with MF3 and the second half with water resulted in much reduction of TMV disease symptoms on both halves. MF3 protein exhibits no adversary effect on the pathogens. Incubation of *Alternaria longipes* spore culture with pure MF3 protein neither affected spore germination nor hyphae growth. Also incubation of viral preparations with MF3 did not influence TMV virulence [8,9]. The molecular analysis of MF3 revealed that its gene is highly homologous to FK506-binding proteins (FKBPs) belonging to the family of peptidyl-prolyl *cis–trans* isomerases [8]. Although the exact function of FKBPs is not completely known, there is evidence that they act in regulation of developmental processes in animals and plants. FKBPs are involved in cell signaling, protein trafficking and transcription [10]. Thus, the homology of MF3 to FKBPs may suggest that MF3 protein takes part in signaling pathway-affecting ISR.

The development of biotechnological methods for genetic modification of higher plants has become a valuable tool for studying plant–microbe interactions and the development of resistant plants [11]. Genetic transformation was also successfully used for the production of carrot with enhanced non-specific resistance to pathogenic fungi by the introduction of pathogenesis-related genes [12]. Tobacco chitinase (TbCH1) increased carrot tolerance to *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotium rolfsii* [13]. Additional enhancement was observed by combining two genes: tobacco *TbCh1* and β-1,3-glucanase [14] or barley chitinase (*chi-2*) and wheat lipid-transfer protein (*ltp*) [15]. A broad spectrum resistance, to *Alternaria* sp., *B. cinerea*, *R. solani* and *Sclerotinia sclerotiorum*, was obtained in carrots expressing rice thaumatin-like protein (*tlp*) [16]. Also human lysozyme enhanced carrot resistance to *Alternaria dauci* and *Erysiphe heraclei* [17]. Here, we report for the first time the successful transfer of the bacterial gene, which expression affected carrot resistance.

In this paper, we report the development of carrot plants with introduced bacterial *mf3* gene using *Agrobacterium*-mediated transformation to evaluate the effect of MF3 protein on carrot resistance when constitutively expressed *in planta*. The results of resistance assays indicate that the transgenic plants had significantly enhanced tolerance to three fungal phytopathogens used, *A. dauci*, *Alternaria radicina* and *B. cinerea*.

2. Materials and methods

2.1. Plant material

Carrot (*Daucus carota* ssp. *sativus* L.) hybrid 'Kamila' was used for transformation procedure to introduce MF3 gene into its genome and then to evaluate its reaction to pathogen invasion. Plants of four other carrot genotypes were used for comparison of resistance reaction: (1) cv. 'Koral', (2) susceptible to *A. dauci* YEL inbred line, (3) tolerant to *A. dauci* cv. 'Bolero' F_1 and (4) tolerant to *A. dauci* wild carrot subspecies *D. c. commutatus*. These genotypes were produced from seed and grown in a glasshouse to get well-developed plants ready at the same time as 'Kamila' plants obtained after regeneration *in vitro* and acclimatization to growth in the glasshouse.

2.2. Vector

Plasmid pLH9000 with inserted *mf3* gene in *Agrobacterium tumefaciens* strain EHA105 cells was constructed by J. Schubert (Institute of Resistance Research and Pathogen Diagnostics, BAZ, Quedlinburg, Germany). The plasmid T-DNA contained 713 bp *mf3* gene (kindly provided by V. Dzhavakhiya, All Russian Research Institute of Phytopathology, Russia) under the control of CaMV 35S promoter and terminator and inserted proximal to the right border. The construct contained neomycin phosphotransferase (*nptII*) gene conferring kanamycin resistance controlled by 35S promoter and terminator. *A. tumefaciens* inoculum was prepared from a bacterial culture grown overnight at 25 °C in YEB [18] medium with 100 mg l^{-1} spectinomycin. The cells were centrifuged at 15,000 rpm for 5 min, washed in ZSMI medium, a modified B5 Gamborg et al. [19] medium (B5 salts, 500 mg l^{-1} KNO_3, 250 mg l^{-1} casein hydrolysate, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamin, 0.1 mg l^{-1} pyridoxin, 20 g l^{-1} sucrose and 0.4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.7) and diluted to $OD_{600} = 0.5$.

2.3. Transformation

Carrot 'Kamila' cell suspension was maintained in ZSMI medium at 25 °C in dark. For transformation, a renewed, 20 ml of 5-day-old cell suspension was incubated with 100 µl of bacterial inoculum for 48 h in dark. Then 400 mg l^{-1} cefotaxim was added for 24 h to kill the bacteria. The suspension was centrifuged at 1000 rpm for 5 min, washed twice with liquid CPPD medium [20] with 0.1 mg l^{-1} naphthalene acetic acid (NAA) and 0.2 mg l^{-1} zeatine and again centrifuged. The collected cells were spread on a filter paper placed on agar CPPD medium with 400 mg l^{-1} cefotaxim and further incubated at 25 °C in dark. After 2 weeks, the filter paper was transferred to B5+ medium (Gamborg B5 salts and vitamins, 500 mg l^{-1} KNO_3, 250 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, 1 mg l^{-1} 2,4-D and 0.02 mg l^{-1} kinetin, pH 5.7)