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Specific Maceration and Induction of *PR-3* Gene in Potato Tuber Tissue by *Pectobacterium carotovorum* subsp. *Atrosepticum* Type III Secretion System Mutants

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Abstract: The exact function of type III secretion system in some phytopathogenes including *Pectobacterium carotovorum* subsp. *atrosepticum* (*Pca*) is not understood and is a matter of debate. The aim of this study were to determine specific effect of type III secretion system on potato tubers and to reveal the connection of this system with potato resistant genes such as *PR-3*. A *Pca hrpW* fragment was subcloned into a low-copy-number cloning vector (pZH448). The resulting plasmid (pAS19) was then conjugated into the wild-type and mutant strains of *Pca* by type III secretion system. The virulence property of different *Pca* strains was studied and the influence of over expression of *hrpW* on maceration activity was also investigated. Furthermore, the effect of mentioned mutation on the maceration of carrot-root was evaluated. Finally, using real-time PCR, the copy-number of *PR-3* gene in potato tuber tissue was assessed. In conclusion, for type III secretion system mutant strains, in contrast with the wild-type, the maceration amount of potato tuber tissue decreased after over expression of *hrpW* while inoculation of tubers by mutants, increased this amount. In the case of potato, HrpN and DspE proteins appeared to be avirulent factors. Compared with the wild-type strains, *Pca* nominated mutants significantly reduced potato *PR-3* expression thus, *PR-3* expression level in potato tuber tissue in answer to infiltration by *Pca*, depends on functional type III secretion system in the bacterium.

Key words: Potato, maceration, *PR-3* gene, *Pectobacterium carotovorum* subsp. *atrosepticum*, type III secretion system

INTRODUCTION

Pca is a commercially important pathogen; it causes blackleg in the field and soft rot of tubers after harvest. The main virulence factors for pectolytic species of *Pectobacteria* (formerly named *Erwinia*) *are* extra cellular depolymerase enzymes including pectolytic, cellulolytic, proteolytic and some others which their roles are not shown (Barras, 1994). Using bacterial depolymerases, *Pca* macerates different plant tissues such as the edible part of carrot root, beets and other plant tissue cultures especially potato, which could do quite a lot of damage to crops (Pérombelon and Salmond, 1995). Recently, scientists have been more interested in the study of type III secretion system (TTSS), including proteins such as harpins or hrp (hypersensitive reaction and pathogenicity) and substrates of this system (Bell *et al*., 2002). The main reason is that the TTSS genes play a role in induction of hypersensitivity reactions apart from the secretion system (Lagonenko *et al*., 2004, 2006; Nicholaichik *et al*., 2005). Although, the role and function of harpins are not clearly shown, it is suggested that they perform as helper in the translocation of Avr-proteins (Alfano and Collmer, 1997). Moreover, they are necessary for the transportation of virulence proteins from bacteria into plant cells (Collmer *et al*., 2002; Wei *et al*., 2000). There are several harpins in different phytopathogenes and their role in bacterial virulence, suppose to be discovered soon (Nisseinen *et al*., 2007). It is also interesting that one of harpin components (HrpW) C-end is similar to pectatelyase in structure (Kim and Beer, 1998).

Plants are known to produce numerous antimicrobial proteins to defend themselves against pathogens and the expression of pathogenesis-related (*PR*) genes being a ubiquitous defense response of plants to pathogen attack (Dangl and Jones, 2001; Nimchuk *et al*., 2003; Rushton and Somssich, 1998). In many plant species, resistance against infections by pathogenic bacteria, viruses and fungi can be induced by a variety of biotic and abiotic elicitors (Ouchi, 1983; Sequeira, 1983). Typically, a pathogen interacting with a resistant host plant triggers a localized hypersensitive response, the intensity and spread of which are regulated by complex molecular mechanisms (Baker *et al*., 1997). At the same time, long-distance signals initiated at the site of infection leads to the induction of specific PR genes in uninfected

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parts of the plant by means of a process termed systemic acquired resistance or SAR (Ryals *et al*., 1994). The accumulation of PR proteins occurs after pathogen attack at a faster rate in incompatible rather than compatible interactions. In some dicots, such as *tobacco* and *Arabidopsis thaliana*, the induction of PR-proteins tightly correlates with the onset of a broad-spectrum, systemic resistance, generally designated systemic acquired resistance (Ryals *et al*., 1996). In spite of this, recently the differential expression of potato defense response gene, infected by *Erwinia carotovora* was shown (Yasser *et al*., 2008).

In this report, the specific role of *Pca* TTSS genes on maceration of potato tuber tissue was demonstrated. The *PR-3* expression amount of potato tuber tissue, infiltrated by different *Pca* strains was also evaluated.

MATERIALS AND METHODS

The bacterial experiments were conducted in Molecular biology Department of Biology Faculty in Belarus State University from 2006-2009. All bacteria were obtained from bacterial collection of Molecular Biology Department. Potato tubers were obtained from Institute Kartofelovodstva of Belarus academy of sciences. Real-time PCR procedures were carried out in both Department of Molecular Biology of Biology Faculty in Belarus state university and Institute Epidemiology and Microbiology of Belarus academy of sciences.

Bacterial strains, plasmids and culture conditions: Bacterial cultures (Table 1) were stored in test-tubes containing 5 mL of 0.5% meat-pepton agar under sterile Vaseline oil at 4°C. Bacteria were grown either in Lauria and Bertani (LB, Tripton 10 g L^{-1}), Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1} liquid, minimal medium A (Miller, 1976) or agar solidified media. *Pca* and *E. coli* cells were grown at 28 and 37°C, respectively. Liquid cultures were grown in a shaking incubator. When required, antibiotics were added at the following concentrations: ampicillin 100 mg mL^{-1}, gentamicin 10 mg mL^{-1}.

Recombinant DNA and RNA techniques: Preparations of plasmid DNA, restriction enzyme digestion, ligations, DNA electrophoresis and transformations, conjugation as well as western and dot blotting were carried out as described by Sambrook and Ausubel (2001) and Ausubel *et al*. (1992). Total RNA was extracted by means of the TRIZOL method (Bio-Rad Laboratories). The concentration of RNA was estimated spectrophotometrically. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instruction.

Macerating assay: For measuring the degree of maceration, each potato slice of about 1 cm thickness and 2-3 cm^2 surface area, was infiltrated with 5 μL of overnight bacterial cultures (OD about 2.0) at 100% relative humidity at 28°C. Samples were examined after 24 h. The degree of tissue maceration was estimated by determining the case with which the tissue could be pulled apart using a spatula. The macerated tissue of each slice was then weighted.

Real-time PCR: Amplification of *EF-1* and *PR-3* genes was achieved by using two oligonucleotide primers pairs (for*EF-1* -5'-TTGATGCTCTTGACCAGATTAACG-3', 5'-ACGGGCACAGTTCCAATACC-3') and (for *PR-3* 5'-AATAAGCCATCATGCCACAACG-3', 5'- GCAGTATT CGGACCCATCCC-3'). Amplification was performed with the following protocol: 1 cycle at 94°C for 5 min. followed by 34 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec, finalized by 1 cycle at 72°C for 7 min. Amplification was carried out on programmable ABI Prism 7000 (Applied Biosystems). The specificity of PCR products was determined by a SYBR Green1 melting curve. 0.5 μg cDNA was added to the RT-PCR mixture containing 1X PCR buffer (Sigma), 0.2 mM dNTP, 50 nM Primers, 0.2U *Taq* polymerase, 1X SYBR Green1, 2.5 mM $MgCl_2$, 0.5 μg cDNA and up to a final volume of 30 μL dH_2O.

Table 1: Bacterial strains and plasmids used in current work

Strains	Characteristics	Source
JN42	Rif^r. Cm^r. Tn9	Collection of laboratory
JN504	*hrpN*::pJP5603. *hrpW*::Ω$^{sp/sm}$ r^{-} $^{nlR.\ CmR.}$ (Tn9)	Collection of laboratory
TA85	JN42 *hrpJ*::pJP5603	Collection of laboratory
TA5	JN42 *hrpL*::ω	Collection of laboratory
JN502	JN42 *hrpN*::pJP5603; Km^r	Collection of laboratory
VKE	JN42 *dspE*:: pJP5603	Collection of laboratory
HW1	JN42 *hrpW*::Ω$^{sp/sm}$	Collection of laboratory
VKW	JN42 *hrpW*::pJP5603; Km^r	Collection of laboratory
3311	JN42 *rsmA* mt^{-} rif^{R} Km^{R} (TnS)	Collection of laboratory
13A	Wild type extracted from potato stem	Collection of laboratory
pZH448	Gm^r: PJQ200mp18Δ *Cla* I-*Hind* III	Collection of laboratory
pAS19	Insertion of *hrpW* fragment from pLA15 restricted by *BamH*I and *Dra*I into pZH448 restricted by *Sma*I and *BamH*I	Subcloned in this work
pLA15	Insertion of *hrpW* fragment into pFLAG-CTC restricted by *Hind*III and *Sal*I	Collection of laboratory

Statistical analysis: Experiments were done several times and data were statistically analyzed using mean averages and their confidential intervals ($p<0.05$).

RESULTS

Maceration of potato tuber tissue by *hrpW* transformed strains of *Pca*: To examine the effect of transformed *Pca* carrying pAS19 on potato, the degree of potato tuber maceration, after infiltration by the cells was measured (Table 2). It can be seen from the table that, in transformed mutants, the maceration amounts of potato tuber tissue after infiltration decreased compared with non-transformed ones. In transformed wild-type strains, a reduction in maceration amounts was not noted. Strains transformed by vector (without *hrpW*) did not show any differences in maceration amounts compared with non-transformed ones (not shown). Maceration activities varied also in different wild types such as JN42, 13A while *Pca* strain JN42 macerated potato slice more effectively. Table 2 shows that, maceration amount of all strains (mutants and wild-type) were about the same and there were not noted significantly differences in obtained data.

Maceration of potato tuber tissue infiltrated by *Pca*: As there were also some *Pca* constructed TTSS mutants in our laboratory, we were interested in studying the TTSS mutation effect on the maceration of potato tuber tissue. Thus maceration amount tubers infiltrated by non-transformed normal and mutant strains of *Pca* was evaluated (Table 3). All the above mentioned strains effectively destroyed potato tuber tissue during the day after infection, but the amounts of tissue maceration or maceration activity of wild-type and mutants were significantly different. It was concluded that, great amount of potato tuber macerated by *dspE* and *hrpN Pca* mutant strains, happened to be a good test for identification of potato resistant brands against disruption.

Maceration of carrot-root infiltrated by *Pca*: In answering to the question, whether a nominated effect on maceration of potato tissue is specified or not, an experiment was carried out on carrot root by the same strains. There were not shown considerable differences between carrot slices infiltrated by different strains of *Pca* (Table 4).

Induction of potato *PR-3* expression by *Pca* strains: On the basis of mentioned information we measured *PR-3* expression amount in potato tuber tissue infiltrated by different strains of *Pca* (Table 5). It was shown that, maximum amount of resistant *PR-3* gene expression connected to tubers infiltrated by JN42 (normal strain). Meanwhile, minimum amount of nominated gene expression was obtained with mutant strains of VKE and JN502. Data shown in this part of the research confirmed previous results obtained for maceration amount of *Pca* strains (Table 2).

Table 2: Maceration amount of transformed and non-transformed strains of *Pca*

Strains	Optical density (600 nm)	Maceration amount (macerated tissue in mg)
VKE	1.800	810.0±15
VKE+pAS19	1.750	537.5±22
3311	1.650	812.5±58
3311+pAS19	1.600	717.5±34
VKW	1.500	535.0±10
VKW+pAS19	1.450	297.5±38
TA85	1.600	327.5±20
TA85+pAS19	1.550	242.5±29
JN502	1.700	395.0±47
JN502+pAS19	1.680	365.0±11
HW1	1.640	375.0±11
HW1+pAS19	1.650	152.5±18
TA5	1.450	345.0±14
TA5+pAS19	1.450	332.5±15
JN42	1.525	340.0±24
JN42+pAS19	1.510	370.0±19
13A	1.500	645.0±10
13A+pAS19	1.510	672.5±22

Table 3: Maceration amount of potato tuber tissue infiltrated by *Pca* strains

Strains	Optical density (600 nm)	Maceration amount (macerated tissue in mg)
TA85	1.90	405.0±15.5
VKE	2.00	612.5±34.4
JN504	1.85	600.0±39.2
JN42	2.00	357.5±14.9
TA5	2.10	297.5±51.0
HW1	1.95	465.0±29.0
VKW	1.90	477.5±35.6
JN502	2.00	657.5±54.2

Table 4: Maceration amount of carrot root infiltrated by *Pca* strains

Strains	Optical density (600 nm)	Maceration amount (macerated tissue in mg)
TA85	1.90	427.5±37.7
VKE	2.00	495.0±40.9
JN504	1.85	432.5±17.5
JN42	2.00	455.0±39.6
TA5	2.10	485.0±48.3
HW1	1.95	445.0±45.1
VKW	1.90	450.0±18.7
JN502	2.00	450.0±10.8

Table 5: Expression amount of potato tuber tissue *PR-3* infiltrated by *Pca*

Strains	The amount of resistant *PR-3* gene compared with *EF-1α* gene expression (the number of gene copy)
JN42	33.24±2.10
VKE	4.110±0.50
JN504	7.870±1.30
TA5	5.680±1.40
HW1	12.79±0.60
VKW	13.68±1.50
JN502	4.260±0.30
TA85	10.42±1.50

DISCUSSION

Maceration of potato tuber tissue by *wild, hrpW* transformed and mutant strains of *Pca*: Induction of hypersensitivity reaction in *Pca,* was related to TTSS function, therefore it was suggested that one or more proteins secreted by TTSS are responsible for such phenotypic effect as a result of interaction between host-plant and pathogen (Chernov *et al*., 1991). Data presented in this report shows that, over expression of *hrpW* in *Pca* TTSS mutants leads to a reduction in the maceration amount, while this effect is not considered for wild-type strains. Although it was previously shown that over expression of *hrpW* in *E.coli* as a result, formed hypersensitivity reaction in *Nicotiana tabacum* after inoculation (Lagonenko *et al*., 2006).

In the case of potato tuber tissue, for all mutant strains, regarding TTSS genes, (strains JN504, HW1, JN502, TA85, VKE) it seemed that, there was a great increase in maceration activity (between 60-130%) comparing with a native strain JN42 (Table 3).

Maceration of potato tuber tissue infiltrated by *Pca*: In JN502 and HW1 mutants, HrpN and HrpW synthesis damaged respectively, while JN504 strain, was defective in both harpins synthesis. We expected that *hrpN* and *hrpW* mutations in *Pca* decreased bacterial virulence activity and as a result decreased maceration of plant tissues because these mutants in previously experiments reduced hypersensitivity reaction (Lagonenko *et al*., 2004, 2006). As Harpins are necessary for induction of hypersensitivity reaction; it was supposed that maceration activity in case of mutant strains strengths because they were not able to induce hypersensitivity response reaction. Data obtained for *Pca* mutants (TA85 and VKE) in which maceration activity increased, confirms our hypothesis.

Maceration of carrot-root infiltrated by *Pca*: JN42 strain is a normal strain which macerated potato slices significantly less than mutants such as VKE, TA85, 502. As *hrpJ, dspE* è *hrpN* mutant strains of *Pca* increased the bacterial ability for potato tuber disruption and carrot-root maceration amounts were not changed; it seems that HrpN and DspE proteins in the case of potato appear to be a virulence factors. This result (specific virulence effect by *Pca*) is supported with the information previously reported (Pérombelon and Salmond, 1995; Bell *et al*., 2004).

Induction of potato *PR-3* expression by *Pca* strains: It was shown before that, expression of PR-proteins in potato tuber tissue induces by *Pca* pectolytic enzymes (Vidal *et al*., 1997). In the case of tissues infiltrated by JN502 mutant strain, the amount of *PR*-3 expression was about 7 times lower than that in the normal strain (JN42) and this amount in the other mutant strain (VKE); nearly 8 times lower than the one in JN42. For two other mutants of HW1 and VKW (these strains being mutants by *hrpW*), the amount of *PR-3* gene expression, compared with the normal strain, were about 2.6 and 2.4 times lower (corresponded). Thus, it is concluded that, bacterial TTSS directly affected the potato *PR3*-gene expression of potato. Yasser *et al.* (2008) showed similar result, measuring expression level of potato *PR*-3 regulator gene, during the infection by *Erwinia carotovora.*

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