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Virulence Function of Pectobacterium atrosepticum Secretion System Mutants on Evaluation of Some *Solanum tuberosum* Resistance Genes

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ABSTRACT

Background: P.atrosepticum is a commercially important pathogen. It causes blackleg in the field and soft rot of tubers after the harvest. This effect is due to secretion of depolymerases and other virulence factors by several mechanisms including T3SS
Objectives: The effect of bacterial T3SS on Solanumtuberosum (S. tuberosum) varieties and its relationship with S. tuberosum resistance gene expression were studied.
Materials and Methods: A P. atrosepticum HrpW gene was cloned, sequenced and constructed a phylogenic tree with some phytopathogen. The virulence properties of P. atrosepticum strains were investigated and then S. tuberosum varieties were tested for their sensitivity against P. atrosepticum. PR-5 and HIN genes copy-number for infiltrated S. tuberosum tubers were assessed.
Results: The results show that infiltrated tubers of P. atrosepticum T3SS mutants were significantly more macerated than the wild type ones.
Conclusions: PR-5 and HIN expression amounts were depended on bacterial T3SS function.

Keywords: HIN; Hrp; Maceration; Phylogenetic Tree; PR-5; T3SS; T3SS Mutant

1. Background

In some bacterial phytopathogens including *Pectobacteriumatrosepticum* (*P. atrosepticum*), the function of type III secretion systems (T3SS) has not been clearly understood. *P. atrosepticum* is a bacterial pathogen which is restricted to *S. tuberosum* in temperate regions. It causes a blackleg in the field and a soft rot in tubers after harvesting. Extracellular depolymerase enzymes are the main virulence factors for pectolytic species of Pectobacteria, although there are others whose roles have not been determined yet (1). Currently, scientists (2) have been more interested in the study of T3SS and the proteins and substrates of this system because T3SS genes apart from a secretion system play a role in the induction of hypersensitivity reactions (3, 4). Harpin proteins (Hrp) have a unique ability to induce hypersensitivity reactions in the leaflet tissues of tobacco after infiltration and this is connected to the T3SS function (5). HrpN from *Erwinia amylavora* was the first phytopathogenic protein for which T3SS role was shown (6). It is suggested that harpins perform as help-

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Implication for health policy/practice/research/medical education:
As secretion mechanisms in P. atrosepticum is similar to secretion mechanisms in human pathogens, consequently data obtained in this research could be counted as a good background for research projects in medicine.

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ers in the translocation of Avr-proteins but their exact role and function is still not clear (7, 8). A structural similarity has also been shown between the HrpW C-terminal protein and the pectate lyase enzyme (9). In spite of this, it has been supposed that harpins are necessary for the transportation of virulence proteins from bacteria into plant cells (10, 11). Resistance against bacterial infection in plants can be induced by a variety of elicitors (12, 13). Production of antimicrobial proteins such as pathogenesis-related (PR) proteins is a ubiquitous defense response of plants to pathogenic attack (14-16). While interacting with a resistant host plant, pathogens trigger localized hypersensitive responses which are regulated by complex mechanisms (17). Signals initiated at the site of infection lead to the induction of specific PR genes in uninfected parts of the plant by the means of a process termed 'systemic acquired resistance' or SAR (18). Induction of PR-proteins in some dicots tightly correlates with the onset of generally designated systemic acquired resistance (19). PR5 proteins are also called thaumatin-like proteins because of their striking sequence similarity to thaumatin, a sweet-tasting protein from *Thaumatococcusdaniellii* (20). PR5 proteins have been characterized from a wide range of plant species in both dicotyledonous and monocotyledonous plants. HIN is as an exemplary gene for the analysis of elicitor-induced PR gene. HIN is up-regulated during the hypersensitive response, is harpin-induced and generated by an incompatible plant-pathogen interaction (21).

2. Objectives

The objectives of the study were to investigate the effect of T3SS from *P. atrosepticum* on the expression of the resistance genes in *S. tuberosum* and thereby the influenced pathogen virulence or disease severity.

3. Materials and Methods

After the sequencing of *P. atrosepticum* HrpW fragment, the encoded protein was predicted and a phylogenetic tree for some phytopathogens was constructed. Also, virulence properties of *P. atrosepticum* strains and sensitivity of *S. tuberosum* varieties against the bacterium were investigated by assessing the PR-5 and HIN gene expression changes by real-time PCR. Bacterial cultures (*Table 1*) were stored in test-tubes containing 5 mL of 0.5% meat-peptone 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 (22) or agar solidified media. *P. atrosepticum* and *E. coli* cells were grown at 28°C and 37°C, respectively. Bacteria in liquid cultures were grown in a shaking incubator. When required, antibiotics were added at the following concentrations: ampicillin 100 $mg.mL^{-1}$, gentamicin 10 $mg.mL^{-1}$. Preparation of plasmid DNA, restriction enzyme digestion, ligation, DNA electrophoresis and transformation, conjugation as well as western and dot blotting were carried out as previously described (23, 24). pAS17 was constructed by the insertion of the 600 bps HrpW fragment from pLA16 and was digested by BglII and EcoRV and inserted into pUC18 which was restricted by SmaI and BamHI. pAS18 was constructed by the insertion of the 1000 bps HrpW fragment from pLA16; digested by SalI and BglII and inserted into pUC18 restricted by SalI and BamHI. Two subclones of pAS17 (including 600 bps HrpW fragment from *P. atrosepticum*) and pAS18 (including 1000 bps HrpW fragment from *P. atrosepticum*) were used as plasmids carrying HrpW parts for nucleotide sequence determination by Amersham Pharmacia Biotech (UK) apparatus and Oligo-nucleotide primers and Fermentas kit (Lithuania) complex. DNA sequence analyses were performed with the PC gene software (ALFwin Software). The tree was constructed by the comparison of the HrpW sequences in various phytopathogenic bacteria using the Neighbor-joining method (25). The phylogenetic analysis was performed using the MEGA 4 software, which employed 1000 bootstrap replications and the maximum parsimony method. For measuring the degree of maceration, a *S. tuberosum* slice of about 1 cm thickness and 2 - 3 cm^2 surface area was incubated overnight with 5 μL of bacterial cultures (OD about 2.0) at 100% and relative humidity at 28°C. Samples were studied after 24 h. The degree of tissue maceration was estimated by determining the ease at which the tissue could be pulled apart using a spatula. The macerated tissue of each slice was then weighted. Total RNA was extracted by the means of the TRIZOL method (Bio-Rad Laboratories). One gram of tissue was ground in a mortar and pestle. Tissue was put into a plastic screw-cap tube and centrifuged with 15 mL TRIzol reagent and was incubated for 5 min at room temperature or 60° C. It was washed with chloroform and finally, RNA precipitated by adding isopropanol. cDNA was synthesized using a Revert and Aid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions (http://products.invitrogen.com/ivgn/product/15596026). The following RNA/primer mixture was prepared in each tube: 5 μg of total RNA, 3 μL random hexamer (50 $ng.L^{-1}$), 1 μL of 10 mM dNTP mix, DEPC H2O to 20 μL, 4 μL of 5X RT buffer, 0.5 μL of 25 RNase inhibitor, 2 μL of RT enzyme. The tubes were incubated at 42°C for 60 min then heated at 70°C for 15 min and stored at -20°C until use for real-time PCR. 0.5 μg of cDNA was added to the RT-PCR mixture containing 1X PCR buffer (Sigma), 0.2 mM dNTP, 50 nM Primers, 0.2 U Taq polymerase, 1X SYBR Green1, 2.5 mM MgCl 2 and dH 2 O up to a final volume of 30 μL. Amplification of EF-1, PR-5 and HIN genes were achieved by using the following primers: For EF-1 -5'-TTGATGCTCTTGACCAGATTAACG-3', 5'-ACGGGCACAGTTCCAATACC-3'; for PR5 *S. tuberosum* (Osmotin): PR5f -5'-ATTTGAGGTCCATAACAACTGTCC-3', PR5r-5'-CAATTTAGTACGACCCCAAATACG-3' and for HIN: HIN1f -5'-GCAACTGCATTTTCCAAATCATC-3', HIN1r-5'-CACGTAGAAATTGACCTTGTTAGG-3'. Gene-specific forward and reverse primer pair concentrations were

normalized and mixed (concentration of each primer in the mixture was 5 pmol.μL $^{-1}$). Amplification was performed with the following protocol by programmable ABI Prism 7000 (Applied Biosystems): 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. The specificity of the PCR products were determined by a SYBR Green1 melting curve. The real-time PCR results were analyzed by the SDS 7000 software. Experiments were in triplets and data were statistically analyzed using mean averages and their confidence intervals ($P < 0.05$). pLA16 and pUC18 were used as HrpW donor and vector for sub cloning, respectively. HrpW gene was divided into two parts and each part was cloned individually. Both plasmids were transformed into *E. coli* and the inserted fragments were sequenced. A phylogenic tree was constructed by summarizing the results and on the basis of BLAST data searches for HrpW in *P. atrosepticum* and some other phytopathogenic bacteria (25). The effect of T3SS wild type and mutant on *S. tuberosum* tuber was investigated by maceration of the infiltrated tissues in some varieties of *S. tuberosum* tubers including Briz, Adretta, Zhuravinka, Lasunak, and Skarb.

4. Results

All strains effectively destroyed *S. tuberosum* tubers but the extent of tissue maceration between wild-type and mutants was significantly different. Apart from the influence of T3SS mutation on maceration amounts, comparison of maceration of tubers also revealed differences in the degree of sensitivity of various types of *S. tuberosum* (*Table 2*). Also, some other varieties were infiltrated with the normal strain (as a control) and two of the most forceful strains of T3SS mutants (*Table 3*). The results show that the use of T3SS mutation can be a useful marker for detecting sensitivity of different varieties of *S. tuberosum* . As T3SS components can elicit a hypersensitivity response, it seems that the expression of hypersensitivity genes might explain the greater amounts of tuber maceration. Therefore, the degree of PR-5 (osmotin) expression was studied in *S. tuberosum* tubers infiltrated by different strains of *P. atrosepticum* (*Table 4*). The greater amount of PR-5 expression was associated with mutant strains of *P. atrosepticum* rather than the wild type (JN42). As the HIN gene is related to hypersensitivity response and is closely related to the PR-5, its gene expression was studied (7) (*Table 5*).

Table 1. Bacterial Strains and Plasmids Which Were Used in the Current Work

Strains	Characteristics	Source
JN42	Rif^r. Cm^r. Tn9	Collection of Laboratory
JN504	HrpN:pJP5603. HrpW:Ω$^{sp/sm}$r-rifRCmR(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
pLA16	Insertion of HrpW fragment into pFLAG-CTS restricted by HindIII and SalI	Collection of Laboratory
pAS17	Insertion of 600 bpHrpW fragment from pLA16 digested by BglII and EcoRV into pUC18 restricted by SmaI and BamHI	Sub cloned in this work
pAS18	Insertion of 1000 bpHrpW fragment from pLA16 restricted bySalI and BglII into pUC18 restricted by SalI and BamHI	Sub cloned in this work

Table 2. Evaluation of maceration amounts for potato varieties, infiltrated by T3SS mutants and wild type of *P. atrosepticum*

Strains	Optical Density (600 nm)	Maceration Amounts ,Mean ± SD, mg			
	Briz	Zhuravinka	Lasunak	Skarb	VKE
1.9	490.5 ± 23	612.5 ± 34	290.5 ± 11	237.5 ± 10	JN502
1.85	475.5 ± 18	657.5 ± 35	273.5 ± 10	257.5 ± 15	JN504
1.9	392.5 ± 25	600 ± 39.2	245.5 ± 15	222.5 ± 5	HW1
2.1	350 ± 14	465 ± 29.0	215.5 ± 12	205.5 ± 10	VKW
2.0	337.5 ± 25	477.5 ± 35.6	190 ± 9	185.5 ± 7	TA5
1.95	295 ± 20	297.5 ± 51.0	172.5 ± 5	152.5 ± 8	TA85
2.0	265 ± 12	405 ± 15.5	160 ± 10	170.5 ± 5	JN42
2.0	245 ± 5	257.5 ± 10	145 ± 8	135.5 ± 5	

Table 3. Evaluation of Maceration Amounts for Some Potato Tuber Varieties Infiltrated by the Most Strengthen HrpW Mutants and Wild Type of P. atrosepticum

Potato Varieties	Maceration Amount, Mean ± SD, mg		
	JN42	VKW	JN502
Arkhideya	307.5 ± 25	530 ± 20	710 ± 25
Zhuravinka	257.5 ± 10	477.5 ± 35	657.5 ± 35
Briz	245 ± 5	337.5 ± 25	475.5 ± 18
Adretta	217.5 ± 10	270 ± 15	340 ± 20
Lasunak	142.5 ± 5	187 ± 4	265 ± 5
Skarb	135.5 ± 5	185.5 ± 7	257.5 ± 15
Delfin	72.5 ± 8	120 ± 12	190 ± 10
Uladar	45 ± 5	90 ± 5	140 ± 14

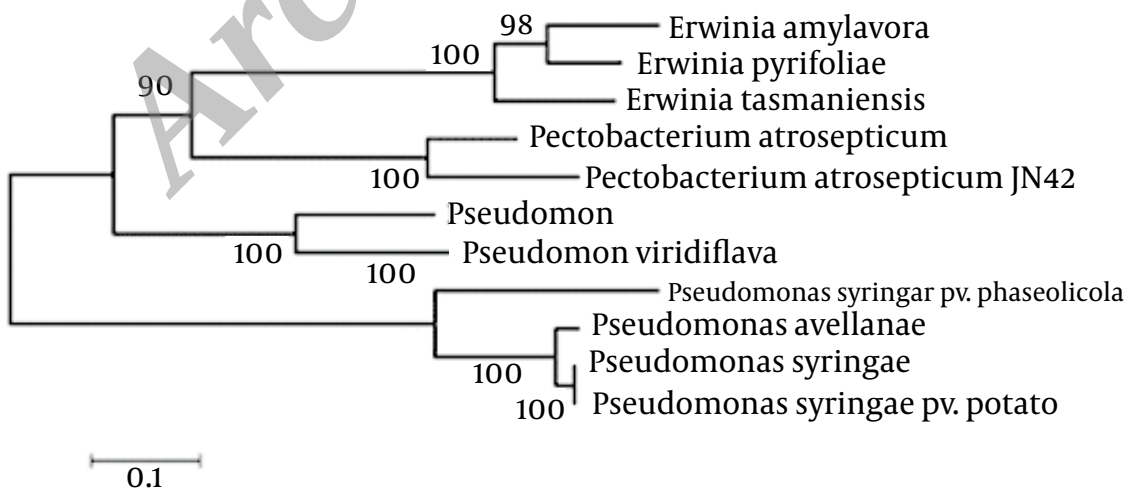
Table 4. Real-Time PCR Evaluation of PR-5 Expression (Gene Copy Number) Regarding EF-1α in Potato Tubers Infiltrated by *P. atrosepticum*

Strains	Expression Level of PR-5 Gene Regarding EF-1 α in Potato Tuber, Mean ± SD
JN42	0.28 ± 0.02
VKE	0.65 ± 0.07
JN504	0.99 ± 0.07
TA5	0.47 ± 0.02
HW1	1.31 ± 0.04
VKW	1.46 ± 0.10
JN502	1.07 ± 0.05
TA85	1.02 ± 0.08

Table 5. Real-time PCR Evaluation of HIN Expression (Gene Copy Number) Regarding EF-1α in Potato Tubers Infiltrated by *P. atrosepticum*

Strains	Expression Level of HIN Gene Regarding EF-1 α In Potato Tuber, Mean ± SD
JN42	17.51 ± 2.90
VKE	36.87 ± 3.10
JN504	50.19 ± 4.20
TA5	42.26 ± 6.40
HW1	92.49± 6.30
VKW	94.94± 8.60
JN502	33.53 ± 4.10
TA85	21.74± 2.50

Figure 1. Constructed Phylogenetic Tree on the Basis of HrpW Protein Sequence



Numbers show the size of bootstrap in percent during 1000 replication.

5. Discussion

HrpW protein characteristics have been demonstrated by previous work (26). Moreover, the complete genomic sequences of the *P. atrosepticum* strain SCRI1043 (Eca1043) and assessment of its similarity to other members of the Enterobacteriaceae and other phytopathogenic bacteria has been described (27); There is a higher similarity between harpins and the cloned HrpW sequence of *P. atrosepticum* strain (JN42) and its encoded proteins (as shown by BLAST data-base search). A phylogenetic tree was constructed by the comparison of HrpW in JN42 strain and similar proteins in other phytopathogens (25) (*Figure 1*). A previously constructed phylogenetic tree based on the 16S rDNA revealed varying levels of taxonomic congruence for the structure of Erwinia, Brenneria and Pectobacterium (26). Moreover, based upon the predicted HrpW sequences, a phylogenetic tree was constructed. It was concluded that the bacterial group of Pectobacteria is divided into pectolytic and nonpectolytic types. PR-5 and HIN expression amounts in *S. tuberosum* tubers were dependent on bacterial T3SS function. It was suggested that HrpN and DspE proteins seemed to be avirulent factors. Considering the high reliability of the present phylogenetic tree, it is possible to divide phytopathogens into three groups. The first group includes *Pseudomonas syringae* and *Pseudomonas avellanae* while the second involves *Pseudomonas cichorii* and *Pseudomonas viridiflava* but *Pectobacterium* falls into the third group. Studies have suggested that hypersensitivity reactions in *P. atrosepticum* may be related to T3SS function (3, 4, 28) as it's been suggested that one or more proteins secreted by T3SS were responsible for this phenotypic effect (as a result of interaction between host-plant and pathogen). There was a considerable increase in maceration activity (60 - 130%) in all T3SS mutant strains (JN504, HW1, JN502, TA85, VKE) compared with the wild type strain (JN42) (*Tables 2* and *Table 3*). This was supported by previous reports (27 , 29). It had been expected that the HrpN and HrpW mutations in *P. atrosepticum* would decrease bacterial virulence because they have already been shown to reduce hypersensitivity reactions (*Table 3* and *Table 4*). To examine whether *S. tuberosum* varieties play role in the amount of maceration, we collected five accessible varieties (Briz, Zhuravinka, Adretta, Lasunak and Skarb) and infiltrated them with *P. atrosepticum* strains. The results showed that, Arkhideya and Zhuravinka varieties of *S. tuberosum* were more sensitive while the least vulnerable ones seemed to be Delfin and Uladar (*Table 3*). Over expression of PR-protein in *S. tuberosum* tuber tissue by *P. atrosepticum* pectolytic enzymes was shown previously (30). Moreover, the influence of *Pectobacterium carotovorum* on PR-3 regulator gene of *S. tuberosum* has also been reported (31). Due to the PR-5 over expression (*Table 4*) in tubers affected by T3SS mutant strains of bacteria compared with the wild type (JN42), it seemed that high-level of expression of PR5 in *S. tuberosum* tubers was related to T3SS. In spite of this, in tubers infiltrated by HrpW mutants (including HW1 and VKW strains) PR-5 expression is significantly higher than the others (*Table 4*). Also, HIN expression in tubers infiltrated by T3SS mutants is higher than the wild-type (*Table 5*). Therefore, It was suggested that high amounts of maceration for infiltrated tubers by T3SS mutants appeared to be a useful marker for the identification of resistant tuber varieties, as *S. tuberosum* slices were significantly less macerated by JN42 strain than T3SS mutants such as VKE and JN502. In addition it was concluded that HrpN and DspE proteins had avirulence activity (*Table 1*).

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Authors' Contribution

This work was done by contribution of Potato Research Institution in Belarus.

Financial Disclosure

Research was done on the basis of biological engineering and safety in Belarus.

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اثر ویرولانسی باکتریهای جهش یافته *P. atrosepticum* از نظرسیستم ترشحی برروی مقدار بیان شدن بعضی از ژنهای مقاومت دهنده *Solanum tuberosum*

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خلاصه مقاله

مقدمه: دربعضی از فیتوباکتریها از جمله *P. atrosepticum* عملکرد سیستم ترشحی نوع سوم به خوبی شناخته نشده است . باکتری نامبرده باعث گندیدگی گیاه سیب زمینی به خصوص در مناطق گرمسیری می شود. ازجمله عوامل موثر در عملکرد این فیتوپاتوژن آنزیمهای دپلیمریزه کننده و نیز فاکتورهای ویرولانسی می باشند.

اهداف: در این مقاله تاثیر سیستم ترشحی نوع سوم یا T3SS بر روی گونه های مختلف گیاه سیب زمینی یا *Solanum tuberosum* (*S. tuberosum*) و ارتباط این سیستم با بیان شدن ژنهای مقاومت دهنده سیب زمینی بر علیه باکتریهای مهاجم بررسی شده است.

مواد و روش ها: ابتدا ژن hrpW مربوط به *P. atrosepticum* کلون و سپس تعیین توالی شد و در نهایت یک درخت فیلوژنتیک برای مقایسه باکتری یاد شده نسبت به سایر فیتوپاتوژنها ترسیم شد. خواص وویژگیهای ویرولانسی سویه های مختلف *P. atrosepticum* مورد سنجش قرار گرفته و در مرحله بعد ی گونه های مختلف سیب زمینی از نظر مقدار حساسیت یا مقاومت در برابر باکتری مربوطه آزمایش شدند. ژنهای 5-PR و HIN براساس تعداد نسخه های تولید شده در سیب زمینی های آلوده شده به باکتری یاد شده مورد بررسی قرار گرفتند.

یافته ها: نتایج بدست آمده نشان داد که برشهای سیب زمینی آلوده شده به *P. atrosepticum* جهش یافته از نظر ژنهای T3SS نسبت به سویه های طبیعی به مراتب بیشتر باعث فاسد شدن این برشها می شوند. ضمنا معلوم شد که بیان شدن ژنهای HIN و 5-PR به عملکرد T3SS بستگی دارد.

بحث و نتیجه گیری: دراین مقاله نشان داده شده است که باکتریهای جهش یافته درژنهای مرتبط با سیستم ترشحی نوع سوم به مراتب بیش از انواع طبیعی باعث گندیدگی سیب زمینی می شوند و نیز اینکه بیان شدن ژنهای ایمنی زای 5-PR و HIN از نظر کمیت بستگی به عملکرد سیستم ترشحی نوع سوم این باکتری دارند.

کاربرد در زمینه سیاست‌های بهداشت و درمان/ پژوهش و آموزش پزشکی:

از آنجا که مکانیسمهای ترشحی در فیتوپاتوژن یاد شده دراین مقاله شباهتهای زیادی با مکانیسمهای ترشحی سموم در پاتوژنهای انسانی هم دارد لذا اطلاعات حاصله می تواند به عنوان زمینه مناسبی برای ادامه تحقیقات برروی پاتوژنهای انسانی هم درنظر گرفته شود.

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