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 phytopathogens 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 (Percwmbelon and Salmon, 1995). Recently, scientists have been more interested in the study of type III secretion system (TTSS), including proteins such as harrpins or hpr (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 harrpins 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 harrpins in different phytopathogens and their role in bacterial virulence, suppose to be discovered soon (Nisseinen et al., 2007). It is also interesting that one of harrpin components (HrpW) C-end is similar to pectate lyase 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
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 cm² 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'-TTGATGCTTGGACACCAGATTACAG-3', 5'-ACGCGCACTAGTCCCATACCC-3') and (for PR-3: 5'-AATAAGCCATCATCACCACACAG-3', 5'-GCAGTATTCCGCACCGATCCCA-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 MgCl₂, 0.5 μg cDNA and up to a final volume of 30 μL dH₂O.

Table 1: Bacterial strains and plasmids used in current work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JN42</td>
<td>Rif, Omp Tn10</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>JN504</td>
<td>hspN:pJP5603, hspW::Omrp + mle Cm á (Tn9)</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>TA85</td>
<td>N42 hsp1-1:pJP5603</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>TA3</td>
<td>N42 hsp1-1 Cm á</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>JN502</td>
<td>N42 hspN:pJP5603, Km1</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>VKE</td>
<td>N42 dmp2-1:pJP5603</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>HWW</td>
<td>N42 hspW::Omrp</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>VKW</td>
<td>N42 hspW:pJP5603, Km1</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>3311</td>
<td>N42 rsm4 mt á rif á Km2 (Tn8)</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>13A</td>
<td>Wild type extracted from potato stem</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>pZH488</td>
<td>Gmr: PJC2000mp180, CiaA-HindIII</td>
<td>Subcloned in this work</td>
</tr>
<tr>
<td>pAS19</td>
<td>Insertion of hspW fragment from pLA15 restricted by BamHI and DdeI into pZH488 restricted by SmaI and BamHI</td>
<td>Collection of laboratory</td>
</tr>
<tr>
<td>pLA15</td>
<td>Insertion of hspW fragment into pFLAG-CTC restricted by HindIII and Sall</td>
<td>Collection of laboratory</td>
</tr>
</tbody>
</table>
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 hrpNPca 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).
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 hrpD, dspE ‘s 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 results, measuring expression level of potato PR-3 regulator gene, during the infection by Erwinia caratovora.

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REFERENCES


