Quantification of Pathogenicity Genes Expression in *Pectobacterium carotovorum* by qRT-PCR

Elfarash, A. E.¹; K. A. Abo Elyousr²; Zynab S. Morsy³ and K. A. Amein¹

¹ Department of Genetics, Faculty of Agriculture, Assiut University, Assiut, Egypt.
² Department of Plant Pathology, Faculty of Agriculture, Assiut University, Assiut, Egypt.
³ Central laboratories, Faculty of Agriculture, Assiut University, Assiut, Egypt.

ABSTRACT

Bacterial soft rot disease is one of the most important and widespread bacterial diseases that cause destructive losses to the crop in a matter of days. The disease causes severe many vegetable crops including potato. In this study, four isolates of *Pectobacterium carotovorum* the main causal of the soft rot disease were isolated from naturally rotted tubers. Then, the isolates were subjected to a new set of primer pairs designed to amplify the *pel* gene which distinguish the *P. carotovorum* strains. The results confirmed that four isolates belong to *P. carotovorum* by having the *pel* gene. Then, a pathogenicity test was performed on potato tubers which showed variability between the isolates in pathogenicity and diseases severity. Finally, a quantitative reverse transcriptase PCR (qRT-PCR) technique was used to investigate the effect of the presence of the potato tissues on stimulating the expression of the pathogenicity genes. The gene expression of five pathogenicity genes was studied included: polygalacturonase (*PeL*), pectate lyase (*Pel*), xylanase (*Xyl*), protease (*Pro*), and cellulase (*Cel*). The results confirmed that the presence of potatoes tissues stimulates the expression of pathogenicity genes in both strong and weak *P. carotovorum* isolates.

**Keywords:** *P. carotovorum*, qRT-PCR, Polygalacturonase, pectate lyase – cellulase, xylanase, protease

INTRODUCTION

Globally, potato is one of the most important food after wheat, rice and maize (Hawkes, 1992). In the field and during storage, many bacterial and fungal diseases can attack the tubers (Wilson, 1960). *Pectobacterium*, certain *Pseudomonas* spp. and *Bacillus* spp are the mainly pathogens of potato (Lelliott et al., 1966). *P. carotovorum* subs*P. carotovorum* is a plant-specific pathogen that cause general plant tissue maceration. The production of plant cell wall degrading enzymes is the main virulence factor of this pathogen. It is producing enzymes, such as: Polygalacturonase, pectate lyase, xylanase, cellulases and proteases, that degrade the plant cell wall (Perombelon, 2002 and Thomson et al., 1999).

Sequencing of several genomes of *P. carotovorum* subs*P. carotovorum* were obtained (Smitis et al., 2010; Park et al., 2011; and Powney et al., 2011). The genomes size ranged from 3.8 to 5.1 Mbp, with coding regions accounted for about 86 % of the total genome, and about 4,263 annotated coding sequences (CDSs) with a length of about 978 bp. The sequencing data confirmed the presence of PCWDEs genes and other pathogenic determinants.

Virulence factors genes like, the producing of extracellular enzymes and others including quorum sensing system, flagellar and chemotactic, type II and III secretion systems genes were highly conserved in the *Pectobacterium* strains (Li et al. 2019).

Real time-PCR is reliable tool that have been applied to many biological processes for molecular detection and quantification (Chin et al., 2008 and Holmes et al., 2006). Since qPCR became available in the 1990s, it has been applied to many different aspects (Zhang and Fang, 2006; N’Guessan et al., 2010 and Sharp et al., 2007).

Targeting functional genes and measuring its gene expression are performed via qRT-PCR, by converting isolated RNA to complementary DNA (cDNA) then quantifying the PCR-amplified DNA (Li et al., 2010).

Recently, qPCR was used on a wide scale to detect viral and bacterial causing potato diseases and estimate the occurrence of some target pathogens (Malko et al., 2019). The analysis of infected leaves and tubers confirmed the dominance of certain potato viruses, *Clavibacter michiganensis* subsp. *sepedonicus*, *P. carotovorum* subs*P. carotovorum*, and *Pectobacterium atrosepticum* in most European regions as Russia.

Ahmed et al. (2017) used the qPCR technique to quantify the *P. carotovorum* bactericidal activity and to evaluate the growth of *P. carotovorum* under the effect of potassium tetraborate tetrahydrate.

Due to the importance *P. carotovorum* in causing an aggressive disease to Potatoes, this investigation was designed to better understand the genetic background of *P. carotovorum* the main causal of the bacterial soft rot disease and to investigate the effect of the presence of potato tissues on stimulating the expression of the pathogenicity genes.

MATERIALS AND METHODS

**Isolation of the causal bacteria.**

Isolation of causal pathogen was carried out from naturally infected potato tubers, with soft rot symptoms collected from Assiut Governorate during 2013-2014 seasons. The infected samples were washed with tap water several times, then the surface was sterilized using in 1%
sodium hypochlorite for two minutes and rinsed twice with water. Then, a small part of the diseased tissues was macerated in 5 ml of 0.05 M potassium phosphate buffer and a loopful of the suspension was streaked onto nutrient sucrose agar medium (Dowson, 1957) and incubated at 28°C for 48 h. Single colony of the isolates was sub-cultured onto slants medium and maintained at 4°C for further studies.

**Pathogenicity tests.**

Isolated bacteria were tested for its ability to cause soft rot disease in both slices (McGuire and Kelman, 1984) and on the full tubers (Yaganza et al., 2003). Four replicates were used for each tested isolate. And all the data were analyzed statistically using L.S.D. test (Gomez and Gomez, 1984).

For the Pathogenicity test on slices, one-centimeter-thick of a sterilized potato slice was kept on a filter paper in Petri-dishes and a loopful of the tested isolates (24 hr. old) was streaked over the slice and incubated at 27°C and observed daily for rotting over a period of 3 days (McGuire and Kelman, 1984).

The pathogenic capability of the tested isolates on the whole tuber were performed by creating a cavity (about 1 cm in depth and 0.5 cm in width) in each tuber by a cork-borer. Then 100 μl of an overnight culture of the tested bacterial isolate were placed in the bottom of the potato cavity. Treated tubers were kept in clean sterilized plastic containers supplemented with a sterilized moist cotton and incubated at 27°C for 4 days. Rotted tubers were weighted and the rotten tissues were removed and weighted again. (Yaganza et al., 2003).

**Expression and quantification of the degrading enzymes by qRT-PCR.**

qRT-PCR experiment was performed to understand the role of degrading enzyme genes in the pathogenic mechanism of *P. carotovorum* by quantifying of the expression of these genes and whether or not the presence of potato tissue in bacterial cultures stimulates expression of pathogenic genes. The expression of several pathogenicity genes was studied included: polygalacturonase (*Peh*), pectate lyase (*Pel*), xylanase (*Xy*), cellulase (*Cel*), and protease (*Prt*). Two isolates, differ in their disease severity, were selected to be used in this study. Isolates were tested under both control (without potato tissue) and induced conditions (potatoes tissues were added to the bacterial cultures).

New sets of primers were designed according to the sequences of the candidate pathogenicity genes (*Peh*, *Pel*, *Cel*, *Xy*, and *Prt*) obtained from the GenBank (https://www.ncbi.nlm.nih.gov/). Sequences of the used primers are listed in Table 1.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer code</th>
<th>Primer Sequence</th>
<th>Band size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polygalacturonase (<em>Peh</em>)</td>
<td>Peh-Fw</td>
<td>TCAGTGTTAAGGVOAGC</td>
<td>277</td>
<td>56</td>
</tr>
<tr>
<td>Pectate lyase (<em>Pel</em>)</td>
<td>Pel2-Fw</td>
<td>GTGACTTACGCCAGTTG</td>
<td>1661</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Pel2-Rv</td>
<td>GCACCAAGCTACGGAAGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulase (<em>Cel</em>)</td>
<td>Cel-Fw</td>
<td>CGTGTTACGAGTGAAGGCT</td>
<td>215</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Cel-Rv</td>
<td>GTAGTGGTTGACGGGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylanase (<em>Xy</em>)</td>
<td>Xy-Fw</td>
<td>CCGTCGCCATCAATCGTGCC</td>
<td>1806</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Xy-Rv</td>
<td>CGCTCAGCGACCACATCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease (<em>Prt</em>)</td>
<td>Prt-Fw</td>
<td>CTCATCGGCATGACGGGAGE</td>
<td>356</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Prt-Rv</td>
<td>ATGGACAGCACAGCTGGCAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total RNA was extracted from three days old bacterial liquid cultures using SV Total RNA Isolation System (Promega) and by following the manufacturer's instructions. Then a DNase treatment using the Max kit from Qiagen was applied to remove residual genomic DNA. RNA concentration and purity were measured in Nano Drop Spectrophotometer ND1000 (Nano Drop Technologies). After that, all RNA samples were diluted to a final concentration of 50 ng/μL and a Reverse transcription for the RNA samples were performed with the first-strand cDNA Synthesis Kit (Amersham Biosciences, GE Healthcare) using pd (N) random hexamers primers.

The qRT-PCR was performed at the Molecular Biology Research Center (MBRU), Assiut University in a Bio-Rad iCycler. The qRT-PCR master mix for each gene was prepared by the following reaction components: 12.5μl iQ SYBR Green Super Mix (Invitrogen), 0.25μl of each primer (10μM), and 8μl nuclease-free water. Then, 4μl of the cDNA were added to a 21μl of the master mix. The following PCR protocol was used: initial denaturation (95°C for 10 minutes), followed by repeated 40 cycles (denaturation: 95°C for 15 sec, annealing: 60°C for 20 sec, 72°C for 60 sec with a single fluorescence measurement), followed by 72°C for 7 min and then cooling to 4°C.

For quantification of gene expression, the cycle threshold (Ct) was determined for each gene transcript. The Relative Quantification (ΔΔCT) method was utilized to calculate fold change (Schmittgen and Livak, 2008). The gene expression of all tested genes was normalized with the absolute expression mean values of the reference gene (*actE*) (Marquez-Villavicencio, et al. 2011).

**RESULTS AND DISCUSSION**

The pathogenicity of the isolates

Four bacterial pure isolates were obtained and tested for its Pathogenicity both potato tubers and slices. All the tested isolates were able to cause maceration of potato slices after 24 hours from inoculation, but they varied in their Disease severity. Data presented in Table (2) indicated that isolate ASY. 7 caused the highest diseases severity of rotted tissues (64.3%) followed by isolates ASY.33 (42.6%) and isolates ASY.26 (34.43%) which caused intermediate disease severity, then isolate ASY.26 was the lowest in disease severity (20.70%).

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Disease severity (%)</th>
<th>L.S.D Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASY 7</td>
<td>64.30</td>
<td>A</td>
</tr>
<tr>
<td>ASY 26</td>
<td>34.43</td>
<td>B</td>
</tr>
<tr>
<td>ASY 33</td>
<td>42.60</td>
<td>B</td>
</tr>
<tr>
<td>ASY 34</td>
<td>20.70</td>
<td>C</td>
</tr>
</tbody>
</table>

Expression and quantification of degrading enzymes genes by qRT-PCR

The qRT-PCR experiment was performed to understand the role of degrading enzyme genes in the pathogenicity of *P. carotovorum* by quantifying of the expression of these genes. And to test whether the presence of potato tissue in bacterial cultures stimulates expression of pathogenic genes or not. 2 isolates were used in this study (isolates ASY 7, 34). These two isolates were selected because they significantly differ in their disease...
severity, since isolate ASY 7 caused the highest diseases severity of rotted tissues while isolate ASY34 showed the lowest diseases severity among the four P. carotovorum isolates (Table 2).

Five candidate pathogenicity genes (Peh, Pel, Cel, Xy, and Prt) were considered for the gene expression analysis using new designed sets of primers. The expressions of these genes were tested under both control (without potato tissue) and induced conditions (potato tissues were added to the bacterial cultures). All experiments were carried out in duplicates. The amplification and melting curves of qRT-PCR of Peh, Pel, Cel, Xy, and Prt genes are represented in Figure (1). The melting–curve analysis was done to confirm specific amplification. The melting–curve results for Cel and Prt genes, showed a single peak profile. This means that no unspecific product was detectable (Figure 1). A few less-sharp peaks in some samples appeared in Peh, Pel and Xy genes which means that they may contain unspecific products, so these contaminated samples were removed and not considered in the statistical analysis.

A relative quantification method calculating the fold difference of a target gene versus a reference gene (dspE gene) were used in all the comparisons according to Pfaffl (2004).

A comparison of the expression fold change of the five genes between the controlled and the induced conditions were made in the isolates ASY7 and ASY34 (Figure 2).

Figure 2. Expression fold change of the different genes showing a comparison between controlled and induced conditions in both (A) isolate ASY 7 and (B) isolate ASY 34.

Isolate ASY 7 showed a higher gene expression levels under the induced conditions than the control treatment in all tested genes (Figure 2A). Similar results were obtained by isolate ASY 34 (Figure 2B) with the Cel, Peh, Pel and Xy genes, but the Prt gene behaved differently since the expression of this gene was higher under the control conditions than the induced conditions. The high expression level of the Prt gene in isolate ASY 34 under the controlled conditions needs further investigation for explanation. With these findings this study could confirm the hypothesis that the presence of potatoes tissues stimulates the expression of pathogenicity genes in both high disease severity (isolates ASY 7) and weak disease severity (isolate ASY 34).

A comparison of the expression fold change of the five genes between isolate ASY 7 and isolate ASY 34 were made under both controlled (Figure 3A) and induced conditions (Figure 3B).

Under the controlled conditions (without adding the potato tissues to the bacterial cultures), all the genes found to be expressed in the two isolates although the absence of the potato’s tissues in the cultures (Figure 3A). This means that the genes were expressed continuously without being hosted on the potato’s tubers but with low expression levels, indicating a potential early role of these genes in the pathogenicity towards the infection of potatoes tubers by soft rot bacteria.

Surprisingly, the data in Figure (3A) shows that isolate ASY 34 expressed the five gene higher than isolate ASY 7 under the controlled conditions (when no potato tissues were added to the bacterial cultures). These data contradict with the disease's severity data (Table 2) since isolate ASY 7 caused higher diseases severity compared to isolate ASY 34. These results means that the genes are expressed continuously and the amount of expression before the infection are not a good indicator for the diseases severity. On the other hand, each strain expressed differently diseases severity during and after infecting the potatoes tubers.

Since isolate ASY 7 caused higher diseases severity and was more aggressive than isolate ASY 34 in infecting the potato tubers (Table 2), so it was expected that isolate ASY 7 will more highly express all the pathogenic genes under the induced conditions (when the potato tissues were added to the

REFERENCES


ASY 34 when compared to isolate ASY 7 (these genes results contradict with the disease severity results). This means that Peh, Prt and Xy genes are playing a higher role than Cel and Pel genes in the infection and development of soft rot disease process in potato’s tubers.

To sum up, our findings confirmed that the tested pathogenicity genes are expressing continuously by P. carotovorum without being hosted on the potato’s tubers but with low levels indicating that these genes are potential for the initiation of the infection. But the amount of expression before the infection are not correlated with the final disease severity which means the presence of other factors affecting disease severity.

After the infection, these genes are highly expressed which means they are stimulated by the potato tissues. In addition, the Peh, Prt, and Xy genes are more important and playing higher role than the Cel, and Pel genes after the infection process of the potato’s tubers. (Oeser et al. (2002) found that Polygalacturonase (Peh) expression is necessary for a successful infection between Claviceps purpurea and rye. Also, Zhang et al. (2005) studies on Rhizoctonia solani demonstrated that polygalacturonase production caused membrane damage, chlorosis, and tissue maceration in rice sheath tissue.

Charkowski et al. (2012) stated that Pectobacterium spp. major virulence factor are the secretion of the plant cell walls degrading enzymes; specifically, pectinases, hemicellulases and proteases. The results obtained in this study under the induced condition (Figure 3B) showed that the expression of the cellulase gene was almost the same in both weak (ASY 34) and strong (ASY 7) tested isolates. Which this result concluded that cellulase is important factor for the infection, but it is not the major factor for diseases severity.

It seems that the regulation of the plant cell wall–degrading enzyme genes (PCWDEs) in P. carotovorum are very complicated. Many of these genes were the master regulator of virulence, although many studies were performed to identify the factors controlling their expression (Burr et al., 2006; Sjöblom et al., 2006 and Deng et al., 2014).

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