Expression of pathogenicity and virulence related genes in *Pseudomonas syringae pv. syringae* under copper stress

Yalda VASEBI1, Reza KHAKVAR1, Mohammad Mehdi FAGHIHI2, Boris Alexander VINATZER3

1University of Tabriz, Faculty of Agriculture
Tabriz, Iran
E-mail: yalda_vasebi@yahoo.com
2Agricultural Research Education and Extension Organization (AREEEO), Hormozgan Agricultural and Natural Resources Research and Education Center
Bandar Abbas, Iran
3Virginia Polytechnic Institute and State University, Virginia Tech School of Plant and Environmental Sciences
Blacksburg, VA 24061, USA

Abstract

Stone fruit bacterial canker is one of the most destructive diseases of apricot in Iran. Copper-based compounds are widely used to protect plants against bacterial diseases, but pathogens frequently evolve resistance against copper (Cu). This study was conducted to investigate the genetic diversity of the Cu resistance gene *cop* among strains of the causal agent of canker disease of stone fruits *Pseudomonas syringae pv. syringae* (Pss), isolated from apricot trees in East Azerbijan province of Iran. The phylogenic trees based on *copA* and *rpoD* are very similar to each other revealing no evidence of recombination. To test the hypothesis that pathogenicity- and virulence-related genes may be induced by Cu in Pss strains, quantitative real-time polymerase chain reaction (qRT-PCR) was used to evaluate the expression of genes *algD*, *copA*, *fhC*, *hrpA*, *syrB* and *tufC*, in the Cu sensitive Pss 170 strain upon exposure to Cu. Among the tested genes, the *algD* and *copA* involved in alginate synthesis and Cu resistance, respectively, showed the highest increase in expression compared to a non-copper (control): 4.75-fold and 2.68-fold, respectively. Based on these results and on the conservation of genes *algD* and *copA* in *Pseudomonas* pathovars, antimicrobials that target AlgD and CopA proteins should be developed to use in combination with Cu to increase control efficiency.

Key words: *algD* gene, *copA* gene, gene expression, real-time PCR, stone fruit canker.

Introduction

*Pseudomonas syringae* strains have been isolated from over 180 plant species (Kennelly et al., 2007). *P. syringae* strains are assigned to more than 50 different pathovars based on host range and 13 phylogroups based on phylogeny (Berge et al., 2014). *P. syringae pv. syringae* (Pss) van Hall is one of the most destructive causal agents of canker diseases on stone fruit trees. The disease causes yield reductions of up to 80% (Kennelly et al., 2007). In Iran, the disease is currently found in most stone fruit orchards in the country (Najafi Pour Haghighi, Taghavi, 2014).

Copper compounds have been widely used for management of bacterial diseases caused by *Pseudomonas* spp., including canker disease of stone fruits (Wimalajeewa et al., 1991). Copper homeostasis systems act as Cu resistance mechanisms and are based on intracellular and extracellular sequestration, enzymatic detoxification, reduced Cu transportation, enhanced efflux of cupric ions, or Cu complexation by cell components (Rademacher, Masepohl, 2012). Essential Cu metabolism-related genes that aid in Cu detoxification are usually located on the main chromosome (Hwang et al., 2005). Copper resistance genes instead can be located either on the main chromosome or on plasmids. Plasmid-determined Cu resistance has been found in diverse Gram-negative bacterial species, such as *P. syringae*, *Xanthomonas campestris* and *Escherichia coli*. In *P. syringae*, plasmid-encoded Cu resistance genes have been identified in various pathovars (Cazorla et al., 2002). *P. syringae pv. tomato* DC3000 was the first intensively-studied Cu resistant plant pathogenic bacterial strain (Cervantes, Gutierrez-Corona, 1994). In this strain, Cu resistance is encoded by the plasmid pPT23D, which is highly conserved among *P. syringae* strains including strains of Pss (Scheck et al., 1996). This plasmid contains a Cu-resistance operon (*copABCDRS*) regulated by a Cu-inducible promoter. Four genes (*copA*, *copB*, *copC* and *copD*) encode structural components and two genes (*copR* and *copS*) have regulatory functions (Mills et al., 1993). *CopA* and *CopB* act as efflux ATPase, while *CopC*...
Expression of pathogenicity and virulence related genes in *Pseudomonas syringae pv. syringae* under copper stress

and *CopD* are responsible for Cu uptake (Rademacher, Masepohl, 2012). Exposure to Cu may affect expression of pathogenicity and virulence genes that encode toxins, such as syringomycin (Scholz-Schroeder et al., 2001). Other virulence genes encode effector proteins that are translocated into plants by the type III secretion system (T3SS), which is encoded by *hrp* genes (Hwang et al., 2005; Ichinose et al., 2013). The *hrp* genes include *hrpA*, which encodes HrpA, the building block of the translocation pilus (Preston et al., 1995). *P. syringae* strains also produce two exopolysaccharides: alginate and levan (Laue et al., 2006). The *algD* is the first gene in the alginate biosynthetic gene cluster in *P. syringae* (Peñalosa-Vázquez et al., 1997). Copper has been found to trigger alginate gene expression in Ps (Kidambi et al., 1995). In fact, alginate can sequestrate Cu ions using electrostatic interactions and keep them trapped outside the cell (González et al., 2010). The twin-arginine translocation (Tat) system also contributes to virulence and pathogenicity (Bronstein et al., 2005). The *tat* operon comprises the tatABC genes. TatC is the most conserved of the Tat proteins and acts as an initial receptor for substrate proteins (Alami et al., 2003). Bronstein et al. (2005) reported that a *tat* mutant strain showed reduced sensitivity to Cu and had attenuated virulence. In strain DC3000, the protein CopA has been identified as substrate of a Tat system, which is transported to the periplasm in a Tat-dependent manner (Bronstein et al., 2005). Finally, the bacterial flagellum mediates adherence, which is another important virulence trait in host colonization and flagellin monomers encoded by the *flIC* gene (Rossez et al., 2015) are important inducers of plant defense responses (Haiko, Westerlund-Wikström, 2013).

The purpose of this study was the investigation of genetic diversity and phylogenetic relationships among *Pseudomonas syringae pv. syringae* (Pss) strains using the *rpoD* housekeeping gene as well as the Cu resistance *copA* gene. Also, the effect of Cu on the expression of a selection of the pathogenicity and virulence-related genes: *algD*, *copA*, *flIC*, *hrpA*, *syrB* and *tatC* was determined in a Cu sensitive Pss strain, which had been identified as a causal agent of canker disease of apricot.

**Materials and methods**

**Isolation and characterization of bacterial strains.** Bacterial strains were isolated from apricot trees in East Azarbaijan province, Iran, in March and April 2015. Tissues that appeared infected, including buds, blossoms, twigs and branches, were selected for isolation. To isolate bacterial strains epiphytically and endophytically, 5 g of crushed tissues were suspended in 20 ml of 0.1 M potassium phosphate buffer and 0.01 M Mg buffer for 1 h and 2 h, respectively, on a shaker at 150 rpm. 100 µL of suspension was streaked on nutrient agar (NA) medium (Merck, Germany) and King’s medium B agar (Biolife, Italy), amended with cyclohexamide (KBC) and incubated at 25°C for three days. Then, morphologically different bacterial colonies were classified using Gram staining and fluorescent production on KB (King’s B) medium. For identification of *P. syringae pv. syringae* (Pss) strains, purified Gram-negative fluorescent colonies were analysed using levan production, oxidase reaction, pectolytic activity on potato slices, arginine dihydrolase activity and hypersensitivity reaction on tobacco leaves (LOPAT test) as well as gelatin liquefaction, tyrosinase activity and tartrate utilization (GATTa test) (Schaad et al., 2001). Syringomycin production and ice nucleation activity were tested according to Schaad et al. (2001).

**Pathogenicity test.** Twenty-four hours old culture of bacterial strains with a concentration of 10⁶ colony forming unit (CFU) ml⁻¹ and sterile double distilled water (DDW) were used in pathogenicity test as positive and negative controls, respectively, on one-year-old apricot twigs. Twigs in leaf germination sites were inoculated with 1 ml of bacterial suspension and then maintained in high moisture conditions at 28°C for 14 days. The presence of necrotic lesions was considered an indication of pathogenetic activity (Moriarty et al., 2001).

**Copper (Cu) resistance and growth curves.** Mannitol-glutamic yeast (Mgy) extract agar is a standard medium used to evaluate Cu resistance *in vitro*. Overnight cultures of each strain on NA medium were suspended in DDW (optical density (OD600) = 0.5). Ten µl of bacterial suspensions in triplicate were spotted on Mgy agar supplemented with filter-sterilized (0.45 µm) stock solutions of Cu (II) sulphate pentahydrate (Merck Millipore, Germany) at 19 concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM. The plates were incubated at 28°C for 72 h. Each assay was performed in triplicate. The minimum inhibitory concentration of copper (MIC-Cu) was defined as the lowest concentration of Cu, at which no growth was observed following incubation at 28°C. Strains with MICs less than 0.75 mM CuSO₄ were scored as Cu sensitive (Cazorla et al., 2002).

**Detection of genes in Pss strains.** CTAB (cetyltrimethylammonium bromide) method according to Doyle and Doyle (1990) with minor modifications was used for total DNA extraction and purification of bacterial strains. A NanoDrop Nano-200 (Allsheng, China) and gel electrophoresis were used for determining of concentration and quality of DNA. The primer pair *rpoD*-F / rpoD-R was used to amplify a fragment of the housekeeping gene rpoD (Sarkar, Gutman, 2004). The primer pair B1/B2 (Sørensen et al., 1998) was used to amplify a fragment of the syringomycin synthesis gene *syrB*. The custom-designed primers PsscopAR/PsscopBF and PsscopAF/PsscopBR were provided by Macrogen Inc. (South Korea) and were used to amplify fragments of internal regions of the *CopD* resistance gene *copB*, respectively. Properties of primers and DNA amplification conditions are summarized in Table 1. All polymerase chain reaction (PCR) assays were performed using a thermocycler Nano-200 (Peqlab Biotechnologie GmbH, Germany) in a final volume of 25 µL containing 12.5 µL of 2x Master Mix Red (AppliQion, Denmark), 10 pmol of each primer for the respective gene, 8.4 µL H₂O and 2 µL of template DNA.

**Sequence alignment and phylogenetic analysis.** PCR products of the *rpoD* and *copA* genes were sequenced in both directions (Macrogen Inc.). The program FinchTV, version 1.4.0 (Geospiza Inc., USA) was used to check the quality of raw sequencing data. Then, software *SeqMan*™ II (DNASTAR Inc., USA) was used for editing and trimming of raw sequencing data. Obtained sequences were used to search the NCBI database (www.ncbi.nlm.nih.gov) using the BLASTN (megablast) tool. The MUSCLE algorithm implemented in the software MEGA 6 (Tamura et al., 2013) was used for sequence aligning. Sequences were aligned using the MUSCLE algorithm implemented in the software MEGA 6 (Tamura et al., 2013).

Phylogenetic trees were constructed using software Mesquite, version 2.75 (Maddison, Maddison, 2011). Phylogenetic analysis was performed using program
MrBayes, version 3.2.2 using a Markov Chain Monte Carlo (MCMC) algorithm (Ronquist et al., 2012) with 100,000,000 generations, average standard deviation of split frequencies value of 0.01, sampling every 1000 generations, and setting of the heating parameter to 0.15. After discarding the first 25% of the generations as "burn-in", the posterior probabilities were calculated from the remaining trees. The Akaike information criterion (AIC) was used to select the best model of nucleotide substitution for each locus. The program FigTree, version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the trees.

**RNA extraction.** A 90 µl inoculum of a culture during exponential growth of the Pss 170 strain in NB medium (Merck, Germany) was added to 70 ml of MGY broth medium without Cu supplementation in a sterile 250 ml Erlenmeyer flask. Bacterial cells were grown in MGY broth medium at 28°C and 150 rpm until sterile. The bacterial cells were harvested during exponential growth of the Pss 170 strain. The bacterial cells were harvested in MGY broth medium at 28°C and 150 rpm until sterile.

**PCR reaction conditions (qRT-PCR).** The PCR reaction mixtures in a total volume of 15 µl contained 7.5 µl of SYBR Green RT PCR Master Mix, 10 pmol of forward and reverse primers and 5 µl of cDNA template. The amplification conditions were as follows: one cycle of initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, at 56°C for 1 min and at 72°C for 1 min. The specificity of all primer pairs and all amplification reactions was confirmed by single-peak melting curve analysis (a denaturing step of 95°C for 15 s, a hybridization step of 1 min at 70°C, followed by temperature increases of 0.3°C per cycle from 70°C to 95°C with a 5 s stop between each step). Gel electrophoresis and melting curve analysis of PCR products showed that very little or no primer dimers were generated. All raw values were normalized to the rpoD gene, which was used as internal reference gene. The relative expression ratios were calculated following the model of Pfaffl (2001), which included an efficiency correction for real-time PCR efficiency of the individual transcripts:

$$\text{Ratio} = \left( \frac{E_{\text{target gene}}^{\Delta Ct}}{E_{\text{reference gene}}^{\Delta Ct}} \right)^{\frac{\Delta Ct_{\text{ref}}(\text{control}) - \Delta Ct_{\text{ref}}(\text{sample})}{\Delta Ct_{\text{target}}(\text{control}) - \Delta Ct_{\text{target}}(\text{sample})}}$$

where E is the real-time PCR efficiency for a given gene, Ct – the crossing point of the amplification curve with the threshold, ΔCt – the crossing point of the amplification curve with the threshold.

### Table 2. Primers for quantitative real-time polymerase chain reaction (qRT-PCR) used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Primer name</th>
<th>Primer sequence 5' to 3'</th>
<th>Amplicon size (bp)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>algD</td>
<td>Alginate synthesis</td>
<td>qRTalgDF, qRTalgDR</td>
<td>GATAGTGGTGCGGCTGGTCTTT, AAGAAGCGCGATCGTGAACTG</td>
<td>90 bp</td>
<td>90%</td>
</tr>
<tr>
<td>copA</td>
<td>Copper resistance</td>
<td>qRTcopAF, qRTcopAR</td>
<td>GATCCATTGGCCGAGGCGTGTG, GCCCACTGACCACTGCCATG</td>
<td>110 bp</td>
<td>90%</td>
</tr>
<tr>
<td>fliC</td>
<td>Flagellin</td>
<td>qRTflicF, qRTflicR</td>
<td>ACCTCGAGATACCAAGGGTCA, AGCCAGATTGACCGATC</td>
<td>125 bp</td>
<td>92.5%</td>
</tr>
<tr>
<td>hrpA</td>
<td>Hrp pilus</td>
<td>qRTHrpAF, qRTHrpAR</td>
<td>GCGTGATTGGCGGAGAT, GCCCTGTTCCTGTTCCTG</td>
<td>130 bp</td>
<td>90%</td>
</tr>
<tr>
<td>rpoD</td>
<td>Sigma factor 70</td>
<td>qRTrpoDF, qRTrpoDR</td>
<td>CCGACGAGGAGAAAGAC, GCCTTTGCGGGTGTATTGCC</td>
<td>101 bp</td>
<td>92.5%</td>
</tr>
<tr>
<td>syrB</td>
<td>Syringomycin synthesis</td>
<td>qRTsyrBF, qRTsyrBR</td>
<td>ACGGTTCGCCGTTGATTGCC, CGACGATGACCCCTGAGAG</td>
<td>129 bp</td>
<td>90%</td>
</tr>
<tr>
<td>tatC</td>
<td>Twin-arginin translocase</td>
<td>qRTtatCF, qRTtatCR</td>
<td>CGAATGAXGACGACGGACAC, AGTGTTGCGGCTGATT</td>
<td>100 bp</td>
<td>90%</td>
</tr>
</tbody>
</table>
difference for an unknown sample versus a control. For each individual gene, the real-time PCR efficiencies were assessed using software LinRegPCR (Rutledge, Stewart, 2008). Amplification efficiencies were determined to be 90% and 92.5%, and were consistent between gene targets. Expression levels of genes are presented as the mean of three replicates. For statistical analysis, data were analysed for variance using ANOVA, and the means were compared by Duncan’s multiple range test using statistical software MSTAT-C, version 1.42 (Michigan State University, USA). Level of significance for different treatments was determined at 5% probability (P < 0.05).

Results

Bacterial isolation and identification. A total of 103 Gram-negative strains were isolated. Five fluorescent and aerobic strains were identified as Pss based on the MIC-Cu – minimum inhibitory concentration of copper

**Copper (Cu) resistance of Pseudomonas syringae pv. syringae (Pss) strains on solid medium.** To assess the antimicrobial activity of copper, MIC-Cu was evaluated. The results showed that the lower concentrations (<0.50 mM) of Cu did not cause any appreciable effect on bacterial growth. However, two strains (Pss 26 and Pss 174) were inhibited by 0.50 mM of Cu and three strains (Pss 82, Pss 170 and Pss 176) were inhibited by 0.75 mM of Cu. No growth was observed for any Pss strains at 1 to 4 mM of Cu.

**Growth of Pss 170 at different concentrations of Cu.** The growth curve of the Cu sensitive Pss 170 strain was determined at two different concentrations (0.50 and 0.75 mM) of Cu in MGY broth medium compared to a control (without Cu). In the absence of Cu, Pss 170 reached the exponential phase (OD600 = 0.6) after 10 h, in the 0.50 mM of Cu, Pss 170 reached the exponential phase (OD600 = 0.6) after 124 h, but in the 0.75 mM of CuSO4 the bacteria stayed in lag phase during the entire 196 h of the experiment (Fig. 1).

**Table 3. Pseudomonas syringae pv. syringae (Pss) strains isolated from infected apricot tissues used in this study**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Geographical areas</th>
<th>Kind of isolation</th>
<th>LOPAT test</th>
<th>GATa test</th>
<th>Pathogenicity test</th>
<th>Ice nucleation activity</th>
<th>Syringomycin production</th>
<th>MIC-Cu</th>
<th>Detection of syB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pss 26</td>
<td>Ajabshir</td>
<td>Epiphytic</td>
<td>++ ++ ++</td>
<td>++ ++ ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.50 mM</td>
<td>+</td>
</tr>
<tr>
<td>Pss 82</td>
<td>Marand</td>
<td>Epiphytic</td>
<td>++ ++ ++</td>
<td>++ ++ ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.75 mM</td>
<td>+</td>
</tr>
<tr>
<td>Pss 170</td>
<td>Sepidan</td>
<td>Epiphytic</td>
<td>++ ++ ++</td>
<td>++ ++ ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.75 mM</td>
<td>+</td>
</tr>
<tr>
<td>Pss 174</td>
<td>Sepidan</td>
<td>Epiphytic</td>
<td>++ ++ ++</td>
<td>++ ++ ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.75 mM</td>
<td>+</td>
</tr>
<tr>
<td>Pss 176</td>
<td>Sepidan</td>
<td>Endophytic</td>
<td>++ ++ ++</td>
<td>++ ++ ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.75 mM</td>
<td>+</td>
</tr>
</tbody>
</table>

**LOPAT** (levan production, oxidase reaction, pectolytic activity and hypersensitivity reaction on tobacco leaves) test (+, −, −, +) and GATa (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and tartrate utilization) test (+, −, −, −). The five strains had been collected from different geographic areas of Iran, including Ajabshir (Pss 26), Marand (Pss 82) and Sepidan (Pss 170, Pss 174 and Pss 176). With the exception of Pss 176, other Pss strains were isolated epiphythically. All five Pss strains produced syringomycin like toxins based on their ability to inhibit mycelial growth of Geotrichum candidum were ice nucleation active and were pathogenic on one-year-old apricot twigs inducing severe necrotic lesions at injection sites (Table 3). Large bacterial populations showing the same colony morphologies were re-isolated from infected tissues and confirmed to be Pss based on biochemical tests and PCR.

**Figure 2.** Agarose 1% gel electrophoresis of PCR products of Pseudomonas syringae pv. syringae (Pss) strains using rpoD-Fp/rpoD-Rp primers

**Figure 3.** Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in Pseudomonas syringae pv. syringae (Pss) strains using B1/B2 primers

**Sequence alignment and phylogenetic analysis.** Partial nucleotide sequences of the rpoD and copA genes obtained from different Cu sensitive strains of Pss were used for phylogenetic analysis. The average sequence length obtained from amplification was 545 and 620 bp for the rpoD and copA gene, respectively. Edited rpoD and copA gene sequences were aligned to the sequences deposited in NCBI’s GenBank database (https://www.ncbi.nlm.nih.gov/). The alignment of the partial nucleotide sequences of the Cu resistance gene revealed homology of over 96% for the copA gene among all reference Pss strains in NCBI’s GenBank.
Figure 4. Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in *Pseudomonas syringae* pv. *syringae* (Pss) strains using PsscopAF/PsscopAR primers.

Figure 5. Agarose 1% gel electrophoresis of PCR-amplified gene coding syringomycin in *Pseudomonas syringae* pv. *syringae* (Pss) strains using PsscopBF/PsscopBR primers.

The best-fit statistical model was determined for each locus based on results from program *MrModeltest* 2.3. General time reversible with gamma distribution (GTR + G) model was recommended for both gene fragments. Phylogenetic trees using Bayesian inference method were constructed to evaluate the evolutionary relationships of the five isolated Pss strains with reference strains based on individual sequences of the *rpoD* (532 nucleotides) and *copA* (599 nucleotides) genes.

The topology of the *copA* gene phylogeny was in agreement with the *rpoD* gene phylogeny. In the phylogenetic trees based on the *rpoD* and *copA* genes, all Iranian Pss strains clustered into two main groups (Fig. 6). Iranian strains in the main group I were divided into two subgroups. Subgroup I included the Pss 170, Pss 174 and Pss 176 strains collected in Sepidan, and subgroup II included the Pss 26 strain collected in Ajabshir. Group II included the Pss 82 strain collected in Marand area. The *Pseudomonas fluorescens* R124 strain was used as outgroup. These biomarkers separated strains collected from the apricot orchards in different geographic areas into different groups and subgroups.

Expression of the selected pathogenicity and virulence-related genes. Because little is known about the regulation of the Cu stress response in Pss strains, the Cu sensitive Pss 170 strain was exposed to elevated concentrations of Cu to examine the responses of pathogenicity and virulence-related genes for up to 12 h using qRT-PCR. The level of relative gene expression was estimated based on the cycle threshold (Ct) values using standard curves. In all cases, the data were normalized relative to the *rpoD* gene. This housekeeping gene was chosen as an internal control, because previous experiments had shown that its transcript levels were not significantly altered under different conditions. Results are shown in Figure 7.

The *algD* gene showed a transcript level range of 0.71 to 4.75 in relative expression. Analysis of results showed that there was a statistically significant difference among treatments at the 5% level (Fig. 3A). In 0.75 and 0.50 mM Cu, *algD* gene expression showed up-regulation of 4.75-fold and 3.4-fold compared to the control (without Cu) after 4 and 12 h, respectively. Analysis of relative expression results of the *copA* gene showed that there was statistical difference among treatments at the 5% level (Fig. 3B). The *copA* gene showed up-regulation in 0.75 and 0.50 mM of Cu after 4 and 12 h compared to the control, respectively. The *flc* gene showed up-regulation most of the time at both Cu concentrations compared to control. Statistical analysis of the ratio obtained from relative expression of the *flc* gene showed that there was difference among treatments at the 5% level (Fig. 3C). The highest increase was 1.85-fold after 12 h of exposure to 0.75 mM Cu compared to the control. For the *hrpA* gene, statistical differences were detected based on the analysis of relative expression among treatments at the 5% level (Fig. 3D). At both Cu concentrations, the highest up regulation (1.3-fold and 1.6-fold in 0.50 and 0.75 mM, respectively) were detected at 2 and 12 h compared to the control. The *syrB* gene showed up-regulation 2.14-fold and 1.6-fold compared to the control in 0.50 and 0.75 mM after 12 and 8 h, respectively, whereby differences were statistically significant among treatments at the 5% level (Fig. 3E). The *syrB* gene showed that there was a statistically significant difference among treatments at the 5% level (Fig. 3F).
Cu and at 4 h in 0.75 mM Cu. A similar up-regulation of regulation compared to control at 12 h in 0.50 mM of the expression level of \( \text{algD} \) gene was 1.93-fold and 1.85-fold, which were obtained after 12 h of Cu exposure compared to control in 0.50 and 0.75 mM Cu, respectively.

Discussion

*Pseudomonas syringae* pv. *syringae* (Pss), the causal agent of bacterial canker of stone fruit, colonizes trees both epiphytically and endophytically. The epiphytic and endophytic phases play important roles in disease epidemiology: the epiphytic population size of the pathogen is associated with disease incidence and severity, because the epiphytic population of Pss on surfaces of apparently healthy blossoms and leaves provides the inoculum for the endophytic phase of the disease. Four of the five Pss strains collected in this study showed ice nucleation activity and thus increase alginate production in a post-exposure to Cu in 0.75 and 0.50 mM Cu, respectively.

CopA is part of the Cu-exporting P-type ATPase and the multicopper oxidase groups. In our study, all Iranian Pss strains clustered with the *P. syringae* reference strains P64, Pss B728a and Pss 301D encoding for P-type ATPase and the multicopper oxidase groups. In our study, all Iranian Pss strains clustered with the *P. syringae* reference strains P64, Pss B728a and Pss 301D encoding for P-type ATPase. CopA is part of the Cu-exporting P-type ATPase IB group of heavy metal transport ATPases (Petersen, Moller, 2000).

The topography of the tree based on partial sequences of the housekeeping gene *rpoD* was similar to the Cu resistance *copA* gene. In the phylogenic tree based on *rpoD*, Iranian strains were grouped the same way as in the *copA* tree. Hwang et al. (2005) reported that the ability of detoxification of Cu appears to be an ancestral trait in *P. syringae* strains. In our study, the presence of *copA* gene in all studied Pss strains agreed with detoxification of Cu being an ancestral trait in *P. syringae*.

Extracellular polysaccharide (EPS) production, such as that of alginate, has been associated with virulence of Pss strains due to its role in increased epiphytic fitness, facilitation of colonization and/or dissemination in planta, induction of water-soaked lesions on infected leaves, adhesion to plant surfaces, biofilm formation and resistance to toxic molecules and dehydration (Yu et al., 1999; O’Brien et al., 2011). Alginate biosynthesis and secretion genes are conserved in the genomes of some Pss strains (Ravindran et al., 2015). In the present study, the highest expression of *algD* gene was found at 4 and 12 h post-exposure to Cu in 0.75 and 0.50 mM Cu, respectively. Our results are consistent with Kidambi et al. (1995) who reported that Cu-based compounds applied for control of bacterial diseases in plants trigger alginate gene expression and thus increase alginate production in a number of Pss strains upon exposure to Cu ions.

In previous research, it had been demonstrated that at high concentrations of Cu, defenses based on the P-type Cu export ATPase are critical virulence factors in pathogenic bacteria (Argüello et al., 2011). In our study, the expression level of *copA* showed an over 2-fold up-regulation compared to control at 12 h in 0.50 mM of Cu and at 4 h in 0.75 mM Cu. A similar up-regulation of...
the Cu resistance genes copA and copB was reported in Xanthomonas axonopodis pv. citri (Palmieri et al., 2010). These results can be explained by the role of these genes in bacterial resistance to antimicrobial compounds, which requires a rapid response to increasing Cu concentrations. The present study is the first to show enhanced expression of virulence-related hrpA, hrc and syrB genes under Cu stress in any P. syringae strain. This is similar to results obtained by Palmieri et al. (2010) for X. axonopodis pv. citri, in which virulence-related (xcsH and xcsC) genes showed enhanced expression in the presence of Cu.

In different bacterial pathogens, participation of the twin-arginine translocation (TAT) system has been shown in both, assembly and function of the flagellum as well as in secretion of cofactor-bound proteins and much virulence factors (Ochsner et al., 2002). In this study, tatC gene expression was up-regulated following inoculation with Cu. Up-regulation of the tatC gene may improve secretion of the Cu resistance protein CopA since previous studies identified the protein CopA as a substrate of the twin-arginine translocation system in strain DC3000 (Bronstein et al., 2005). Also, in the protein PcoA of strain DC3000, participation of the Cu resistance genes copA and copB under Cu stress, their sequence conservation among Pseudomonas strains (Muhammad, Ahmed, 2007) and essential role in defence against Cu suggest that novel antimicrobial compounds could be developed to interfere with the function of proteins AlgD and CopA to increase susceptibility of Pss to Cu.

Conclusions

1. All collected Pseudomonas syringae pv. syringae (Pss) strains were copper (Cu) sensitive with minimum inhibitory concentration (MIC) less than 0.75 mM.
2. In the phylogenic tree based on the copA gene, all Iranian Pss strains clustered with the Pss reference strains encoding for CopA protein as a part of the Cu-exporting p-type ATPase.

Acknowledgement

The authors are grateful for financial support from Iran National Science Foundation (INSF) (grant No. 93005043).
Expression of pathogenicity and virulence related genes in Pseudomonas syringae pv. syringae under copper stress

Y. Vasebi1, R. Khakvar1, M. M. Faghihi2, B. A. Vinatzer3

1Tebriz university
2Iran University of Medical Sciences
3Auburn University

ISSN 1392-3196 / e-ISSN 2335-8947
DOI 10.1371/journal.ppat.1004483

Vario streso įtaka Pseudomonas syringae pv. syringae patogeniškumo ir virulentiškumo genų raiškai

Y. Vasebi1, R. Khakvar1, M. M. Faghihi2, B. A. Vinatzer3

1Tebriz university
2Iran University of Medical Sciences
3Auburn University

ISSN 1392-3196 / e-ISSN 2335-8947
DOI 10.1371/journal.ppat.1004483