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BACTERIAL AND PHYTOPLASMA DISEASES

Oxidative stress regulates the expression of the Pht cluster genes involved in phaseolotoxin synthesis in *Pseudomonas syringae* pv. *phaseolicola* NPS3121

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Abstract

This study evaluated the role of oxidative stress on the expression of Pht cluster genes involved in phaseolotoxin synthesis in *Pseudomonas syringae* pv. *phaseolicola*. Results demonstrate that the expression of Pht cluster genes is regulated by oxidative stress in a manner dependent of the ROS present in the cell. The presence of H_2O_2 and Paraquat, influences on the expression of the Pht cluster genes in function of the compound and of the concentration evaluated, demonstrating that expression of Pht genes is part of the oxidative stress response in *P. syringae* pv. *phaseolicola* NPS3121.

Keywords *Pseudomonas syringae* pv. *phaseolicola* · Phaseolotoxin · Pht cluster · Oxidative stress · Thermoregulation.

Introduction

Pseudomonas syringae pv. *phaseolicola* is the causal agent of the common bean (*Phaseolus vulgaris* L.) disease known as halo blight. This disease is considered one of the most common and economically important bacterial diseases of bean plants because it causes major field crop losses, especially in temperate and cold areas (Singh et al. 1995). Among the virulence factors that the *P. syringae* pv. *phaseolicola* bacterium synthesizes for the development of this disease, highlights the phaseolotoxin production, which is the main responsible of the characteristic chlorotic symptoms of the halo blight disease (Mitchell 1976; Mitchell and Bieleski 1977). Phaseolotoxin is an important element for the development of the disease and the major virulence factor of the pathogen (Ferguson and Johnston 1980; Templeton et al. 1984). Production of phaseolotoxin by *P. syringae* pv. *phaseolicola* is regulated mainly by temperature; low temperatures (18–20 °C) favor the phaseolotoxin synthesis, but the toxin is not detected at 28 °C, the optimal growth temperature for this bacterium (Goss 1970; Nüske and Fritsche 1989).

Genes necessary for the synthesis of this compound have been identified in a chromosomal region called the Pht cluster, which comprises 23 genes organized in five transcriptional units including two monocistronic (*argK* and *phtL*) and three polycistronic operons, one comprising 11 genes from *phtA* to *phtK* with an internal promoter driving expression of *phtD* to *phtK* and a third large polycistronic operon comprising 10 genes from *phtM* to *phtV* (Aguilera et al. 2007). Analysis of gene expression within the Pht cluster showed that most of the genes are regulated at the transcriptional level by low temperatures showing high transcript levels at 18 °C with basal levels of expression at 28 °C (Aguilera et al. 2007). The mechanism by which *P. syringae* pv. *phaseolicola* regulates the expression of these genes in relation to low temperature is poorly understood.

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So far, the signal transduction pathways involved in the integration of low-temperature perception and phaseolotoxin synthesis have not been clarified. Recent studies of the transcriptome comparing the expression profile of the *P. syringae* pv. *phaseolicola* bacterium grown at 28 and 18 °C showed that the global expression profile obtained at low temperatures (18 °C) coincides with an oxidative stress response. Thus, these analyses suggested that the low temperatures could induce an oxidative stress in the bacteria, which in turn gave rise to the gene expression profile obtained, within which is highlighted the expression of the Pht cluster genes (Arvizu-Gómez et al. 2013). In this manner, the Pht cluster genes expression appear to be part of the oxidative stress response in the *P. syringae* pv. *phaseolicola* bacterium. To evaluate whether the expression of the Pht cluster genes is regulated by oxidative stress and whether this is part of the signal transduction pathway involved in phaseolotoxin synthesis, we undertook the present study.

Initially, intracellular oxidative stress was induced in *P. syringae* pv. *phaseolicola* NPS3121. Cultures of the wild type (wt) strain grown at 28 °C as described by Arvizu-Gómez et al. (2013) and under oxidative stress inducer conditions, obtained by the addition of the hydrogen peroxide (H_2O_2) reagent, or the superoxide ion (O_2^-)-generating compound Paraquat were performed. Two concentrations of H_2O_2 (1 and 2.5 mM) and Paraquat (1 and 50 mM) were evaluated. These concentrations known to induce oxidative stress in various *Pseudomonas* species (Cao et al. 2012; Ochsner et al. 2000; Palma et al. 2004), and the generation of an expression profile concentration-dependent (Cao et al. 2012; Katsuwon and Anderson 1992). The cultures, in the presence of the oxidative stress-generating agent, were incubated at 28 °C for 10 min, the necessary incubation time to generate an oxidative stress response in *Pseudomonas* sp. (Chang et al. 2005; Ochsner et al. 2000; Wei et al. 2012). Reactive oxygen species (ROS) generation, characteristics of an oxidative stress condition, was determined by fluorescence through of the oxidation of the fluorescent probe, 2,7-dihydrodichlorofluorescein diacetate (DCFH-DA) as described by Remes et al. (2014). The data analyses showed that the exposure of the cells to H_2O_2 at 1 and 2.5 mM, induced high levels of intracellular ROS in comparison to the control cells (without H_2O_2). Furthermore, this intracellular ROS generation was independent of the H_2O_2 concentration used because no significant difference in ROS content in the cells treated with either concentration (Fig. 1).

For cells exposed to Paraquat, intracellular ROS was generated only at the high concentration of the inducer agent (50 mM) in comparison to the control cells (Fig. 1). Additionally, the data analysis showed that only the cells exposed to high concentrations of H_2O_2 (2.5 mM) differed significantly in the intracellular ROS content from the level induced by Paraquat; thus, exposure to 2.5 mM H_2O_2 is associated with

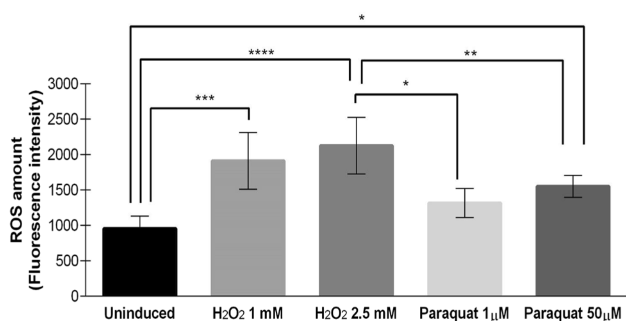

Fig. 1 Intracellular levels of reactive oxygen species (ROS) in *Pseudomonas syringae* pv. *phaseolicola* NPS3121 under inducer conditions of oxidative stress. Cultures from the wild-type (WT) strain were grown under normal conditions, without inducing (black) (Uninduced) and in presence of the oxidative stress inducer agents, H_2O_2 (1 and 2.5 mM) or Paraquat (1 and 50 mM). ROS generation was measured using an oxidation-sensitive fluorescent probe, 2,7-dihydrodichlorofluorescein diacetate (DCFH-DA). The ROS intracellular levels are expressed as fluorescence intensity. Each graphic represent the mean values (SD) of two experiments. ****$P<0.0001$, Uninduced vs 2.5 mM H_2O_2; ***$P=0.0001$, Uninduced vs 1 mM H_2O_2; **$P=0.0085$, 2.5 mM H_2O_2 vs 1 mM Paraquat; *$P<0.05$, 1 mM H_2O_2 vs 1 μM Paraquat and Uninduced vs 50 mM Paraquat

major induction of intracellular oxidative stress (Fig. 1). These results demonstrated the capacity of H_2O_2 and Paraquat at the concentrations evaluated to induce intracellular oxidative stress in *P. syringae* pv. *phaseolicola* NPS3121.

Because the Pht gene cluster had been postulated as being part of the oxidative stress response of *P. syringae* pv. *phaseolicola* NPS3121 (Arvizu-Gómez et al. 2013), we decide to evaluate whether under the conditions used, an oxidative stress response was generated in the bacteria. RT-PCR assays were performed analyzing the expression pattern of the *katB* gene (PSPPH_3274), encoding catalase, which is involved in protecting cells from oxidative damage by ROS (Cabiscol et al. 2000). Total RNA was extracted from the wt strain grown as described above and used in RT-PCR assays with the pair oligonucleotides KatBfw (CTTTGA CTTCTTCTCGCATGTTC) and KatBrv (AGAAGTTGT CGACGTTCTTGTTC) using the Super-Script one-step kit (Invitrogen, California, USA) and 200 ng RNA template per reaction. Controls used for each set of primers were (1) PCR without the reverse transcription step to verify the absence of DNA, (2) RT-PCR performed without RNA templates to detect any contaminating DNA/RNA, (3) PCR performed using genomic DNA as a template to ensure primer fidelity, and (4) amplification of a portion of the 16S rRNA operon as an internal control of the reaction. The RT reaction was performed at 50 °C for 30 min; followed by PCR amplification at 94 °C for 2 min for 1 cycle; 94 °C for 35 s, 50 °C for 30 s, and 72 °C for 1 min for 18–25 cycles; and 72 °C for 10 min for 1 cycle. The results showed high transcript

levels for the *katB* gene in both concentrations of H_2O_2 and Paraquat, in comparison to those obtained in the untreated cells (Fig. 2). The basal transcript levels for *katB* in the control cells (untreated) could be part of the stress response to the accumulation of ROS produced by the metabolism during the cellular growth. The expression of genes encoding catalases in diverse bacteria is upregulated during early stationary growth (González-Flecha and Demple 1997), the growth stage of the bacteria in the present study. These results demonstrate that under the used conditions of H_2O_2 and Paraquat, an oxidative stress response is generated in the *P. syringae* pv. *phaseolicola* bacterium.

Finally, to assess the influence of the oxidative stress induced by H_2O_2 and Paraquat on the expression of the Pht cluster genes, we used RT-PCR under the conditions above mentioned. Five genes (*argK, phtA, desI, phtL* and *amtA*) representatives of the five transcriptional units that make up the Pht cluster were selected and evaluated by these assays (Fig. 2a). In general, the results demonstrated that expression of the Pht cluster genes is regulated by oxidative stress in a manner dependent on the ROS level in the cell. H_2O_2 exposure at 1 mM H_2O_2 resulted in the upregulation of expression of all five Pht cluster genes at 28 °C, in contrast to 2.5 mM, which upregulated two (*phtD* and *phtL*) of the five transcriptional units evaluated (Fig. 2b). Interestingly, the two concentrations of H_2O_2 did not induce any differences in intracellular ROS, despite the differences in the genes that they upregulated. More experimental work is necessary to clarify the reason for this difference. With regards to the presence of the superoxide ion (O_2^-) generating compound, Paraquat, both positive and negative effects on the expression of Pht cluster genes were observed. Additionally, the results demonstrated that the expression pattern obtained in the most of the Pht cluster genes (*desI, phtL*, and *amtA*) is increased in function of the oxidant agent-Paraquat concentration (Fig. 2c). The *desI* and *phtL* genes were downregulated, while the *amtA* gene was upregulated. Expression of the *argK* gene changed only after exposure to 1 µM Paraquat, not to the higher concentration, and the transcript level for *phtA* did not change, regardless of the concentration (Fig. 2c).

Although the thermoregulation of the Pht genes was abolished by the oxidative stress generated by the oxidants, particularly 1 mM H_2O_2, phaseolotoxin was not detected in the medium (data not shown). These results suggest that genes independent of the Pht cluster are involved in the synthesis of phaseolotoxin, and its expression could be mediated by regulatory mechanisms independent of oxidative stress. So far, it is unknown whether only the Pht gene cluster is involved in the phaseolotoxin synthesis, so the participation of other genes cannot be ruled out. The PSPPH_4550 gene, which encodes a putative non-ribosomal peptide synthetase and is not part of the Pht gene cluster, is involved in the synthesis of phaseolotoxin in *P. syringae* pv. *phaseolicola* NPS3121; however, its function has been proposed as related to bacterial fitness (De la Torre-Zavala et al. 2011). Although the expression of the PSPPH_4550 gene is dependent on low temperature, similar to the case of the Pht cluster genes, the influence of oxidative stress on the expression of this gene has not been established.

The fact that specific expression profiles were obtained depending on the oxidant agent used (H_2O_2 or Paraquat) suggests that different regulatory pathways of oxidative stress mediate the expression of the Pht cluster genes. The mechanisms of the oxidative stress response, in which the OxyR and SoxRS regulatory proteins are involved, have been extensively studied and characterized for *Escherichia coli* (Chiang and Schellhorn 2012; Demple 1991; Imlay 2008). In a two-step process, the SoxR protein is initially activated by the oxidation of its Fe-S centers via O_2^- to further enhance the transcription of the SoxS protein. SoxS then activates the expression of the SoxRS regulon. On the other hand, OxyR is activated directly by H_2O_2-mediated disulfide oxidation. Once activated, OxyR induces the expression of genes in the

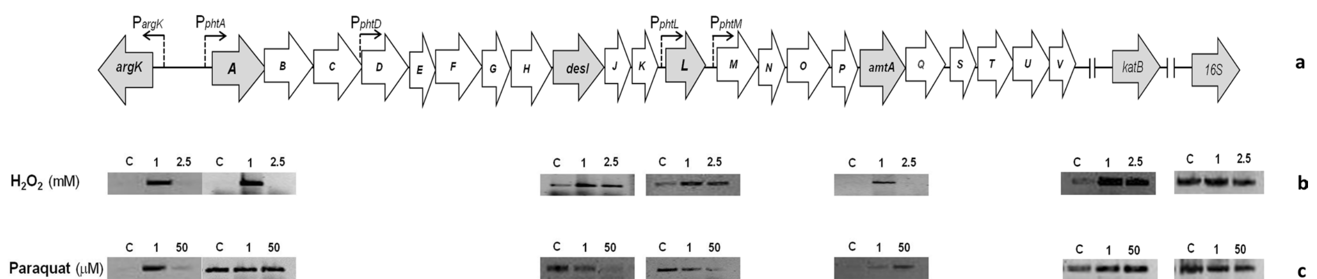

Fig. 2 Expression profile of the oxidative stress response gene *katB* and the Pht cluster genes of *Pseudomonas syringae* pv. *phaseolicola* NPS3121 under oxidative stress. **a** Graphic representation of the Pht cluster and catalase gene. Each arrow represents an individual gene, with the direction of the arrow indicating the direction of transcription. Grays arrows indicate the genes evaluated by RT-PCR analyses. The oxidative stress-generating agents H_2O_2 (1 and 2.5 mM) and Paraquat (1 and 50 mM) were used in this study. **b** Expression patterns obtained after exposure to H_2O_2. *C* untreated cells (control), 1 = 1 mM H_2O_2, 2.5 = 2.5 mM H_2O_2. **c** Expression pattern obtained in the presence of Paraquat. *C* untreated cells (control), 1 = 1 µM Paraquat, 50 = 50 mM

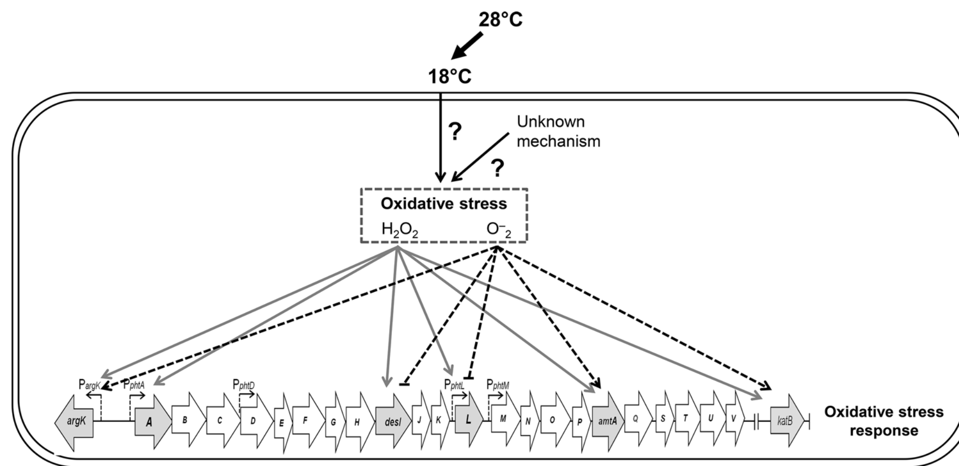

Fig. 3 Oxidative stress as part of the signaling pathway involved in the expression of the Pht cluster genes. Model proposed to explain the influence of oxidative stress on the regulation of Pht cluster genes. Intracellular oxidative stress is generated after the perception of low temperature (18 °C) or by an unknown mechanism. This oxidative stress regulates the expression of Pht cluster genes in a manner dependent on ROS. The gray arrows and dashed lines represent the effect of H_2O_2 and (O_2^-), respectively, on the Pht cluster genes. The arrow indicates a positive effect on expression; the T bar represents inhibition

OxyR regulon (Chiang and Schellhorn 2012; Demple 1991). Studies focused mainly on the oxidative stress response in various bacteria have used H_2O_2 as an oxidizing agent that acts on the OxyR regulator and O_2^--generating compounds, such as Paraquat, which activates the transcription factor SoxR (Chiang and Schellhorn 2012). The OxyR and SoxRS proteins in various bacteria, including *Pseudomonas* species, are functionally conserved (Chiang and Schellhorn 2012). The relation between the presence of oxidative stress and the synthesis of virulence factors in some bacterial pathogens, such as *Pseudomonas aeruginosa* and *Pantoea stewartii*, has already been reported (Burbank and Roper 2014; Pacheco et al. 2012). Furthermore, there is evidence of the regulation of various virulence factors in phytopathogenic bacteria by the mechanisms that regulate response to oxidative stress (OxyR and SoxRS) (Burbank and Roper 2014; Flores-Cruz and Allen 2011; Nachin et al. 2001). Currently, we are investigating the mechanisms by which oxidative stress regulates the expression of the Pht cluster genes as well as the role of the OxyR and SoxRS regulators on the phaseolotoxin synthesis.

On the other hand, the results obtained in this study suggest the existence of various alternative regulatory pathways involved in the expression of the Pht cluster genes; this is based on the previous evidence of regulation related to this process. Previous studies established a regulatory function for the PhtL protein, which positively regulates the expression of the *phtM* operon at 18 °C (Aguilera et al. 2007). Based on the results obtained in this work, the regulatory function previously suggested for the PhtL protein is not congruent with the expression pattern under oxidative stress conditions (with exception of H_2O_2 1 mM) that we obtained. In the presence of 2.5 mM H_2O_2, *phtL* is upregulated at 28 °C but *amtA* (a member of the *phtM* operon) is downregulated. Likewise, the presence of Paraquat (1 and 50 µM) caused a decrease in the transcript levels of *phtL*, but *amtA* was upregulated. Similar results were obtained with respect to the PhtABC proteins, which have been reported as involved in the transcriptional repression of *argK* at 28 °C whose repression is abolished at low temperatures (Aguilera et al. 2012). Our results showed that under oxidative stress, the *phtABC* genes were induced, as was the *argK* gene, similar to the expression at low temperatures (18 °C). The results of this work suggests the participation of other independent regulatory circuits involved in the expression of the Pht cluster genes. Furthermore, this study demonstrates that the oxidative stress is part of the signaling pathways involved in phaseolotoxin synthesis (Fig. 3) and that the expression of the Pht cluster genes is part of the oxidative stress response in *P. syringae* pv. *phaseolicola* NPS3121.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. The authors have full control of all primary data and agree to allow the journal to review the data if requested.

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