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An interpromoter region enhances transcription of *argK* gene, encoding the phaseolotoxin-resistant ornithine carbamoyltransferase in *Pseudomonas syringae* pv. phaseolicola

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Abstract

Pseudomonas syringae pv. phaseolicola, is the causal agent of halo blight on bean, a disease characterized by water soaked lesions surrounded by a chlorotic halo, which is produced by the action of phaseolotoxin, a nonhost-specific toxin optimally produced between 18°C to 20°C. To protect itself against phaseolotoxin, *P. syringae* pv. phaseolicola possesses a phaseolotoxin-resistant OCTase (ROCT) encoded by *argK*, which is expressed under conditions leading to the synthesis of phaseolotoxin. The promoter of *argK* is divergent to that of *phtABC* genes and both share an interpromoter region. The objective of the present study was to evaluate the role of the interpromoter region on *argK* transcription in *P. syringae* pv. phaseolicola. To this, deletions of the interpromoter region were made and *argK* expression was evaluated in an heterologous system. Also, the effect of multiple copies of the interpromoter region on phaseolotoxin production was determined. The obtained results showed that this region is necessary to enhance the transcription of *argK*. Additionally, this region in a multiple copies, interfered with the thermoregulation of phaseolotoxin at 28°C in strain *P. syringae* pv. phaseolicola NPS3121.

Key Words: phaseolotoxin, *argK* gene, promoter, thermoregulation, interpromoter region.

Una región interpromotora incrementa la transcripción del gen *argK*, que codifica para la ornitina carbamoiltransferasa resistente a la faseolotoxina en *Pseudomonas syringae* pv. phaseolicola

Resumen

Pseudomonas syringae pv. phaseolicola, es el agente causal del tizón del halo del frijol, una enfermedad que se caracteriza por presentar lesiones acuosas rodeadas por un halo clorótico, el cual es producido por la acción de la faseolotoxina, una toxina no específica del hospedero, producida de manera óptima entre 18°C a 20°C. Para protegerse de su propia toxina, *P. syringae* pv. phaseolicola posee una ornitina carbamoyl-transferasa (OCTasa) resistente a la faseolotoxina (ROCT) codificada por *argK*, que es expresada bajo las condiciones de síntesis de la faseolotoxina. El promotor de *argK* es divergente al promotor de *phtABC* y ambos comparten una región interpromotora. El objetivo del presente estudio fue evaluar la función de la región interpromotora sobre la transcripción de *argK* en *P. syringae* pv. phaseolicola. Para esto, se llevaron a cabo deleciones de la región interpromotora y se evaluó la transcripción de *argK* en un sistema heterólogo. También se determinó el efecto de múltiples copias de la región interpromotora sobre la producción de la faseolotoxina. Los resultados obtenidos mostraron que esta región es necesaria para incrementar la transcripción de *argK*. Adicionalmente, cuando se ponen múltiples copias de esta región, se interfiere con la termorregulación de la faseolotoxina a 28°C en la cepa de *P. syringae* pv. phaseolicola NPS3121.

Palabras Clave: faseolotoxina, gen *argK*, promotor, termorregulación, región interpromotora.

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Introduction

Pseudomonas syringae pv. phaseolicola infects bean (*Phaseolus vulgaris* L), *P. syringae* pv. actinidiae infects kiwi (*Actinidia chinensis*), and in strain CFBP3388 of *P. syringae* pv. syringae, isolated from vetch (*Vicia sativa*) produce a nonhost-specific, chlorosis inducing toxin known as phaseolotoxin (Mitchell, 1976; Tamura *et al*., 2002; Tourte & Manceau, 1995).

The production of this toxin is temperature dependent, being optimally produced between 18°C to 20°C, while no detectable amounts of phaseolotoxin are present above 28°C (Goss, 1940; Mitchell, 1978; Nuske & Fritsche, 1989). Phaseolotoxin is a reversible inhibitor of the enzymes ornithine carbamoyltransferase (OCTase; EC 2.1.3.3) (Ferguson, Johnston & Mitchell, 1980), which catalyzes the formation of citrulline from ornithine and carbamoylphosphate in the sixth step of the arginine biosynthetic pathway; and ornithine decarboxylase, which participates in the biosynthesis of polyamines (Bachmann, Matile & Slusarenko, 1998). *P. syringae* pv. phaseolicola is insensitive to the effect of its own toxin. The reason for this immunity was attributed to the presence of a phaseolotoxin-resistant OCTase (ROCT) activity in strains of *P. syringae* pv. phaseolicola capable of producing the toxin (Jahn, Sauerstein & Reuter, 1985; Mosqueda *et al*., 1990; Staskawicz, Panopoulo & Hoogenraad, 1980). The ROCT, is a polypeptide composed of 327 amino acid residues with a molecular mass of 36.52 kDa and is the product of the *argK* gene, which is expressed under conditions leading to the synthesis of phaseolotoxin, such as growth in minimal medium at 18°C (Hatziloukas & Panopoulos, 1992; Jahn, Sauerstein & Reuter, 1987; Mosqueda *et al*., 1990). The gene *argK* is necessary for *P. syringae* pv. phaseolicola under conditions of phaseolotoxin synthesis, because it ensures an optimal supply of the arginine required for its growth (López-López, Hernández-Flores, Cruz-Aguilar & Álvarez-Morales, 2004). There is evidence that *argK* is negatively regulated at 28°C by a repressor protein (Mosqueda *et al*., 1990). It was proposed that the repressor protein could binds to a specific DNA motifs (TRR; thermoregulatory region) found in the *argK* interpromoter region, which have been postulated to be involved in thermoregulation of phaseolotoxin synthesis (Rowley, Xu & Patil, 2000). Additionally, it has been shown that *argK* is not directly regulated by temperature, but most likely is regulated by a precursor of phaseolotoxin resembling carbamoylphosphate (López-López, Hernández-Flores, Cruz-Aguilar & Álvarez-Morales, 2004).

The genes required for the biosynthesis of phaseolotoxin are grouped in a genomic region that is called Pht cluster containing 23 genes included into a genomic region (Figure 1A) (Aguilera *et al.*, 2007; Genka *et al*., 2006). These genes are organized in five transcriptional units, two monocistronic and three polycistronic, with one of them overlapping a larger operon (Aguilera *et al*., 2007).

Recently, it was demonstrated that the coordinated participation of *phtA*, *phtB* and *phtC* products, coded into the Pht cluster, are necessary to carry out an efficient *argK* repression at temperatures not permissive for phaseolotoxin biosynthesis. Also, *argK* transcriptional control is mediated by a protein present in both, toxigenic and nontoxigenic strains of *P. syringae* and in *Escherichia coli* (Aguilera *et al*., 2012). The promoters for *argK* and *phtABC* genes have been previously determined and shown to be a Pribnow-type (σ^{70}) promoters with appropriate -10 and -35 regions (Aguilera *et al*., 2007; Hatziloukas & Panopoulos, 1992). The promoter driving the expression of *phtABC* is divergent to the promoter of *argK* and both share an interpromoter region with distinct sequence features that suggest coordinate regulation (Aguilera *et al*., 2007). According to this, the aim of the present study was to evaluate the role of the interpromoter region on *argK* transcription in *P. syringae* pv. phaseolicola. To this end, we deleted this region and analyzed *argK* transcription in an heterologous system. Additionally, the effect of multiple copies of the region on phaseolotoxin production in strain NPS3121 was determined.

Materials and methods

Media and bacterial strains used in this work

The bacterial strains and plasmids used in this study are listed in Table I. *E. coli* DH5α was grown in Luria Bertani (LB) medium at 37°C. *P. syringae* pv. phaseolicola were routinely grown on King´s B medium (King, Ward & Raney, 1954). For phaseolotoxin production, *P. syringae* pv. phaseolicola was grown in M9 medium at 18°C during 48 h (Sambrook, Fritsch & Maniatis, 1989). *P. syringae* pv. phaseolicola NPS3121 and *P. syringae* pv. phaseolicola CYL233 are referred to as wild type strains. When required, carbenicillin was added at 100 (*E. coli*) or 300 (*P. syringae*) µg/ml final concentration. Primer sequences and targets are indicated in Table II and Figure 1B.

Molecular biology techniques

Routine techniques were performed as described previously (Sambrook, Fritsch & Maniatis, 1989). Plasmids and DNA were purified from agarose gels with QIAGEN columns and kits (Valencia, Ca, USA). Chromosomal DNA from *P. syringae* pv. phaseolicola was obtained as described previously (Chen & Kuo, 1993). Restriction enzymes were used according to instructions provided by the suppliers. DNA fragments used as probes for Northern blots were labelled with [α-^{32}P]dCTP using the Rediprime II Random Prime Labeling System (GE Healthcare, Buckinghamshire, UK).

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Table I. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (Φ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nal^r	(Sambrook, Fritsch & Maniatis, 1989)
P. syringae pv. phaseolicola		
NPS3121	Wild type, Tox$^+$	(Peet, Lindgren, Willis & Panopoulos, 1986)
CYL233	Wild type, Tox$^-$	(Rico *et al*., 2003)
Plasmids		
pUCP20	*Pseudomonas-E. coli* shuttle vector; Ap^r; 3.89-kb; *lacZ´*	(West, Schweizer, Dall, Sample & Runyen-Janecky, 1994)
pSAK	Ap^r; SAK in pUCP20	(Aguilera *et al*., 2012)
pKD0	Ap^r; KD0 in pUCP20	This study
pKD1	Ap^r; KD1 in pUCP20	This study
pKD2	Ap^r; KD2 in pUCP20	This study

Table II. Primers used in this study.

Amplicon	Primer Name	Primer sequence (5´→3´)[a]	Restriction enzimes	Reference
Northern blot				
argK	L10001	CTTTGATGGTATGCATGCGGTT		(Aguilera *et al*., 2012)
	L10002	GGAAGAACTGGCCAAACATTCG		(Aguilera *et al*., 2012)
Construction of plasmids				
KD0	L100135	CAATCCGAA**CTGCAG**CCGAGACTAACGAAA	PstI	(Aguilera *et al*., 2012)
	L100276	GGGCAAT**GGATCC**TTGACACGC	BamHI	This study
KD1	L100135	CAATCCGAA**CTGCAG**CCGAGACTAACGAAA	PstI	(Aguilera *et al*., 2012)
	L100172	CGACTAC**CCCGGG**CCTGCACAAG	SmaI	This study
KD2	L100135	CAATCCGAA**CTGCAG**CCGAGACTA ACGAAA	PstI	(Aguilera *et al*., 2012)
	L100170	TGCAAAAACC**CCCGGG**CTACACTA ATG	SmaI	This study

[a]Restriction sites are indicated in boldface.

Construction of plasmids containing *argK* promoter deletions

The genes *phtABC* and *argK* share an interpromoter region (149 bp) comprised among the -35 consensus sequences of each promoter (Figure 1A). Three versions of *argK* containing several interpromoter deletions (KD0, KD1 and KD2) were obtained by PCR using primers designed to include suitable restriction sites (Table II; Figure 1B). The KD0 amplicon was cloned into PstI-BamHI sites of the pUCP20 vector to create pKD0 plasmid. This amplicon contained 5 bp of the interpromoter region. The KD1 and KD2 amplicons were cloned into pUCP20 PstI-SmaI sites to create pKD1 and pKD2 plasmids. Both amplicons contained 35 bp and 78 bp of the interpromoter region, respectively. All constructions were confirmed by restriction patterns and electroporated into *P. syringae* pv. phaseolicola using a BioRad Gene Pulser (Hercules, Ca, USA) with the following parameters: 200 V, 20 Ω y 250 μFD.

RNA extraction and Northern blot analysis

The expression of *argK* was determined by Northern blot analysis. To that end, total RNA was extracted from cells by using TRIzol reagent as recommended by the manufacturer (Invitrogen, Carlsbad, Ca, USA) from cultures of *P. syringae* pv. phaseolicola grown in M9 medium at 18°C or 28°C until an $O.D_{600}$

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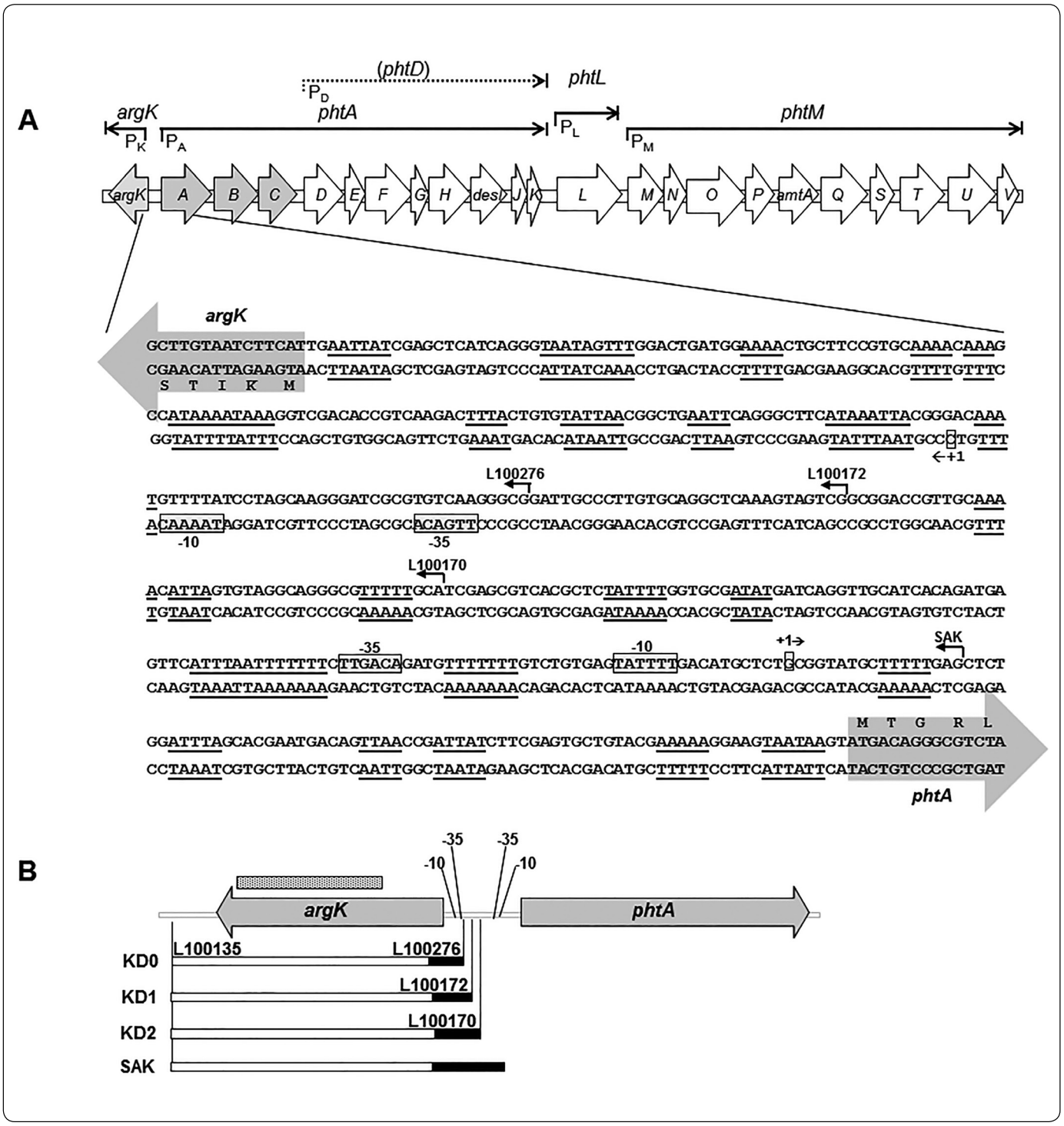

Figure 1. Promoter regions of the *argK* and *phtABC* genes. On top are depicted the Pht cluster of *P. syringae* pv. phaseolicola. The operons are named after the first gene of the operon. In the middle, nucleotide sequence of the *argK-phtA* intergenic region showing the -10 and -35 promoter sites for *argK* and for *phtA* are shown. The double-stranded sequence is shown to facilitate the identification of relevant sequences on both strands as the promoters are divergent. AT-rich sequences are underlined. In the bottom, amplicons and primers for KD0, KD1 and KD2 are depicted as bars. White bars represent the coding region of gene *argK* and black bars, represent the *argK* promoter and deletions. Northern blot probe is indicated as shaded bar above *argK* gene.

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of 0.8. Genomic DNA was removed by digestion with TURBO DNA-free Kit (Invitrogen, Carlsbad, Ca, USA). Samples of total RNA (20 µg) were separated by electrophoresis using 1.3% denaturing agarose gels. The RNA was transferred to Hybond N^+ nylon membranes (GE Healthcare, Buckinghamshire, UK) and cross-linked by exposure to UV radiation. Hybridization was performed using NorthernMax Prehybridization/Hybridization buffer (Ambion, Carlsbad, Ca, USA). A DNA probe (607 bp) for the *argK* gene was obtained using oligonucleotides previously reported (Table II). Hybridization was carried out overnight at 50°C. The membranes were washed twice with 2X SSC-0.1% sodium dodecyl sulfate (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 5 min at room temperature, followed by a wash with 1X SSC-0.1% sodium dodecyl sulfate for 3 min at 60°C. The membranes were exposed to a phosphorus screen and the signal detected in a Storm 860 apparatus (Molecular Dynamics, Sunnyvale, Ca, USA). The image analysis was made using ImageQuant version 1.1 (BioRad, Hercules, Ca, USA).

Phaseolotoxin bioassays

Phaseolotoxin production by *P. syringae* pv. phaseolicola NPS3121 was assayed by the *E. coli* growth inhibition assay as previously was described (Hernández-Guzmán & Álvarez-Morales, 2001). In every case, plates containing arginine were used as controls to confirm that growth inhibition was due to phaseolotoxin.

Results and discussion

Effect of deletions of the interpromoter region on *argK* transcription pattern in *P. syringae* pv. *phaseolicola* CYL233

To assess the effect of interpromoter region on *argK* transcription, we constructed plasmids with three deletions upstream -10 and -35 regions of the *argK* promoter (Figure 1A). Northern blot analysis were made to evaluate the expression of *argK* in an heterologous system. As a positive control expression, strain NPS3121 was used; as a negative control we used the strain CYL233(pUCP20), since strain CYL233 is a *P. syringae* pv. phaseolicola that does not produces phaseolotoxin because it lacks the Pht cluster (Rico *et al*., 2003). Also, we evaluated the transcript expression on strain CYL233(pSAK), which contains the complete interpromoter region.

Our work group has used the strain CYL233 to perform heterologous expression assays of genes from the Pht cluster in a genetic background similar to strain NPS3121. In those studies we have observed that in strain CYL233(pSAK), the introduced *argK* gene is expressed both 18°C and 28°C contrary to strain NPS3121*wt* in which *argK* is expressed only at 18°C (Aguilera *et al*., 2012; Aguilera *et al*., 2007). In this study, we also confirmed the expression of *argK* in CYL233(pSAK) at both temperatures in contrast with the expression at 18°C in the wild type strain NPS3121 (Figure

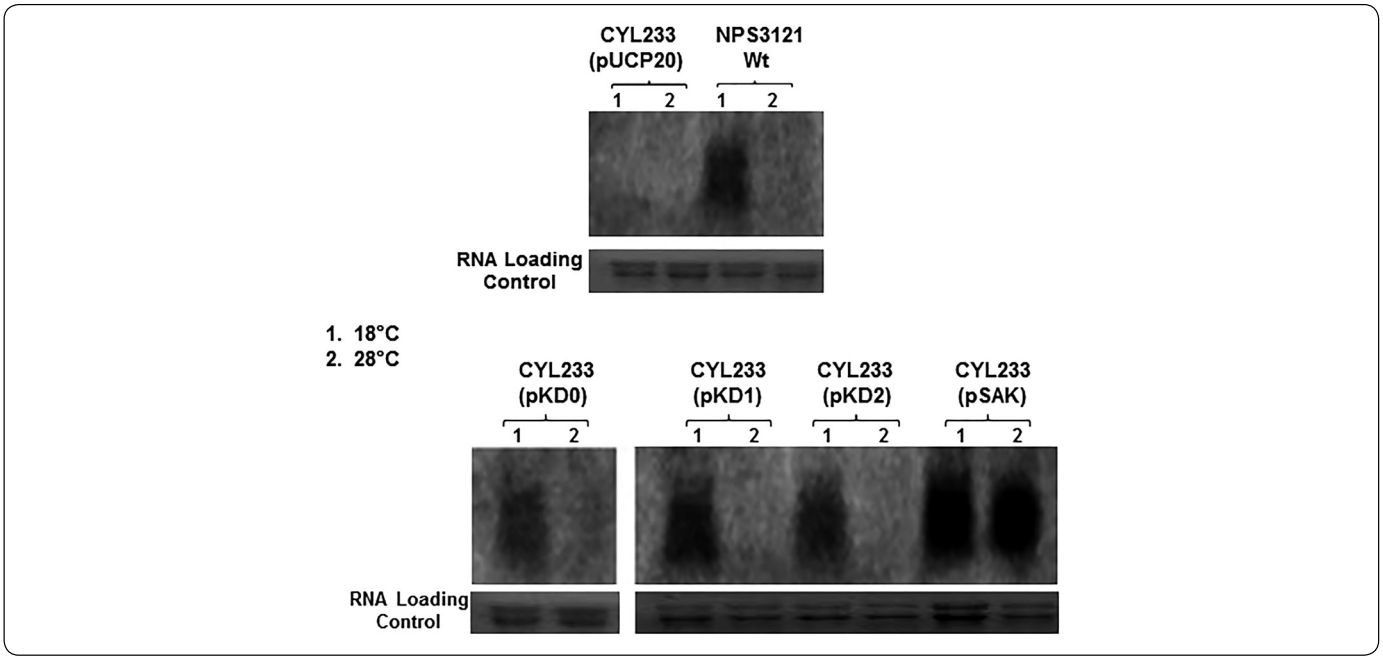

Figure 2. Effect of deletions of interpromoter region on the *argK* expression in *P. syringae* pv. phaseolicola. The expression of *argK* was evaluated by Northern blot in derivatives of strain CYL233 harboring plasmids with deletions as indicated above each blot. Blots were hybridized with an internal probe specific for *argK*, and the signal corresponding to the monocistronic *argK* RNA. Strain CYL233(pUCP20) was used as negative control of *argK* expression, whereas the wild type strain NPS3121 was used as a positive control. The numbers on top of the Northern blots represent the temperatures at which expression was assayed: 1 indicates 18°C and 2 indicates 28°C.

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2). The plasmid pKD0 contains a 144 bp deletion affecting almost completely the interpromoter region, since only 5 bp remains upstream -35 region (Figure 1A). This deletion reduced the *argK* expression at 18°C compared with the strain CYL233(pSAK) containing the complete region at the same temperature.

On the other hand, plasmids pKD1 and pKD2 carry part of the interpromoter sequence in which 114 bp and 71 bp were deleted, respectively. In both cases, we observed expression of *argK* only at 18°C, unlike the expression detected in CYL233(pSAK) at both temperatures. In all cases, *argK* expression is achieved without the need of any element coded by the Pht cluster, in agreement with previous reports (Aguilera *et al*., 2012).

For deletions encoded in plasmids pKD1 and pKD2, we observed that *argK* expression was a little stronger than that observed for the pKD0 plasmid (Figure 2), this may be due to the fact that pKD1 and pKD2 keep a bigger part of the interpromoter region than pKD0. It is important to note that no appreciable difference is observed among expression pattern for plasmids pKD1 and pKD2, suggesting that elimination of 71 bp of the interpromoter region is enough to promote the clearly diminish *argK* transcription and therefore, the sites within the 71 bp are necessary and sufficient for the efficient transcription of *argK*.

It was reported that *argK* and *phtABC* divergent promoters show AT-rich sites on both strands that could be implicated in polymerase binding (Aguilera *et al*., 2007). It is well known that in prokaryotes, RNA polymerase recognizes two consensus sequences in the promoter region sites, known as -10 and -35 boxes (Dombroski, Walter, Record, Siegele & Gross, 1992). These consensus sequences of *argK* promoter are conserved in plasmid pKD0, which allows some level of expression for *argK*, although less efficient when compared with the complete interpromoter region, at 18°C. It has been observed that in some promoters, AT-rich sequences are also recognized by the sigma subunit of the RNA polymerase (Ross *et al*., 1993). The efficiency between the promoter region and the RNA polymerase depends directly on variations in the consensus sequence, as well as the variations in the space among sequences (Dombroski, Walter, Record, Siegele & Gross, 1992). Based in the high affinity of the RNA polymerase for AT-rich sequences, we proposed that the AT-rich sequences located within interpromoter region (Figure 1A) could enhance the activity of the *argK* promoter, interacting with the C-terminus of the RNA polymerase sigma subunit.

It was interesting to note that in strain CYL233 containing the plasmids pKD0, pKD1 or pKD2, the transcription of *argK* at 28°C was abolished, in contrast with the expression observed in the same strain containing the plasmid pSAK (Figure 2). We believe that deleted fragments are important not only for *argK* transcription (as observed at 18°C), but also could be involved in the regulation mediated by temperature.

Effect of multiple copies of the interpromoter region on phaseolotoxin production.

According to our results, we suggest that deleted fragments could be involved in *argK* thermoregulation. It has been proposed that in *P. syringae* pv. phaseolicola, the *argK* gene have TRR (thermoregulatory region) motifs which could be involved in its thermoregulation. Additionally, TRR motifs have been postulated to be involved in the control of phaseolotoxin synthesis (Rowley, Clements, Mandel, Humphreys & Patil, 1993; Rowley, Xu & Patil, 2000). In an effort to investigate more about these regions, we evaluated the effect of multiple copies of the interpromoter region on the phaseolotoxin production in *P. syringae* pv. phaseolicola strain NPS3121. To this, we obtained the strains NPS3121(pSAK) and NPS3121(pKD2), which contain both a chromosomal and the plasmids copies of *argK* and its regulatory sequences. The plasmid pKD2 occurs in multiple copies in a similar way to the plasmid pSAK (Aguilera *et al*., 2012).

As shown by the growth inhibition assay, the strains NPS3121(pSAK) and NPS3121(pKD2) produce phaseolotoxin at a level comparable to that of the wild type strain at 18°C. Unexpectedly, at 28°C, strain NPS3121(pSAK) was able to produce phaseolotoxin, (although at very low level) compared to wild type strain NPS3121 alone or harboring the plasmid pKD2 at the same temperature (Figure 3). It was reported that when TRR motifs, present in multiple copies, could override the thermoregulation of phaseolotoxin production by wild type cells at 28°C (Rowley, Clements, Mandel, Humphreys & Patil, 1993). In a similar way, we observed that the interpromoter region, in a multiple copies, also override the thermoregulation of phaseolotoxin at 28°C. At the moment we do not have a satisfactory explanation about the phaseolotoxin production in strain NPS3121(pSAK) at 28°C, however is plausible that sequences resembling TRR motifs encoded in this plasmid, can titrate the available concentration of the repressor at 28°C, explaining the effect observed. Further work to understand this intriguing and interesting regulatory mechanism is currently under way in our laboratory.

Conclusions

This study revealed the importance of the interpromoter region to enhance the transcription of *argK* in *P. syringae* pv. phaseolicola. Additionally, this region in a multiple copies, interfered with the thermoregulation of phaseolotoxin at 28°C in strain NPS3121. Indeed, regulation of the phaseolotoxin biosynthesis is very complex and there are several unknown regulatory circuits that differentially affect the transcription of *pht* genes, including *argK*.

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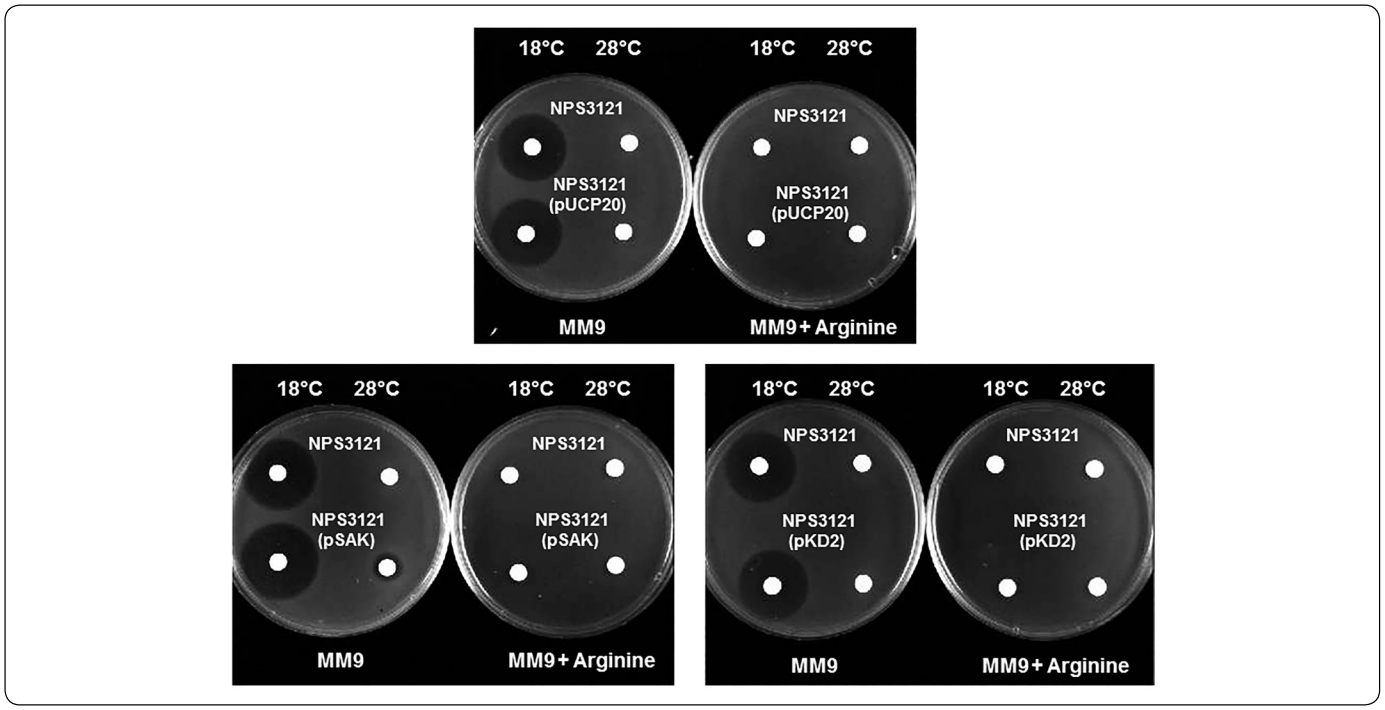

Figure 3. Phaseolotoxin bioassay for *P. syringae* pv. phaseolicola NPS3121 harboring multiple copies of the interpromoter region. *E. coli* JM103 growth inhibition in minimal medium by added phaseolotoxin. Control; minimal medium with phaseolotoxin and supplemented with 10 mM arginine. On the left side of dishes, paper discs contain supernatant of cultures at 18°C; on right side, supernatant of cultures at 28°C.

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