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Heterologous expression of predicted promoter site for paraquat-inducible genes of the bacterium *Chromobacterium violaceum* is increased by plumbagin

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Resumo

Região predita de um promotor de genes induzíveis por paraquat da bactéria *Chromobacterium violaceum* sofre indução heteróloga pelo composto plumbagin. O objetivo deste estudo foi avaliar funcionalmente a influência do composto plumbagin sobre a indução heteróloga de uma região promotora predita de genes paraquat-induzíveis revelada durante as análises de anotação do genoma da bactéria *Chromobacterium violaceum*. A região promotora de interesse de *C. violaceum* foi amplificada a partir de sequências oligonucleotídicas específicas e clonada em vetor conjugativo, sendo acoplada à região codificante do gene *lacZ* de *Escherichia coli*. Em seguida, a indução heteróloga desse segmento regulatório foi estimada em cepa de *E. coli* na presença do plumbagin em uma concentração final de 50 µg/mL por mensurações dos níveis de expressão da enzima β-galactosidase. Diferenças significativas nos níveis da β-galactosidase foram observadas como resultado da ativação da região promotora de interesse pelo plumbagin na concentração testada em comparação às condições controle. Por outro lado, nenhum crescimento da cepa selvagem de *C. violaceum* foi observado durante a incubação dessas células em meio nutritivo contendo diferentes concentrações do plumbagin. As descobertas descritas aqui sugerem que uma região promotora predita para genes paraquat-induzíveis da bactéria *C. violaceum* sofra indução heteróloga pelo plumbagin, reforçando evidências acerca da caracterização funcional de motivos regulatórios intrínsecos a essa bactéria.

Palavras-chave: Agente oxidante; Expressão heteróloga; Genoma funcional

Abstract

The aim of this study was to evaluate functionally the effect of plumbagin on the heterologous expression of a predicted promoter region of open reading frames of paraquat-inducible (*pqi*) genes revealed during genome

annotation analyses of the bacterium *Chromobacterium violaceum*. First, the promoter of interest was amplified using specific primers and cloned into a conjugative vector carrying the *Escherichia coli* lacZ gene without a promoter. The heterologous expression of the predicted promoter region was then examined in the presence of 50 µg/mL plumbagin by β-galactosidase expression assays. Significant differences were detected in the levels of β-galactosidase as a result of the activation of the promoter region of interest in response to plumbagin at the concentration tested. On the other hand, no growth of the wild strain of *C. violaceum* was found during its incubation in nutrient broth medium containing different concentrations of plumbagin compared to control group. The findings described herein demonstrate that the heterologous expression of a predicted promoter site of *pqi* genes of *C. violaceum* is induced by plumbagin in a fusion strain, giving insights into the functional characterization of intrinsic regulatory DNA motifs annotated in this bacterial genome.

Key words: Functional genome; Heterologous expression; Oxidizing agent

Introduction

Chromobacterium violaceum is a Gram-negative, free-living betaproteobacterium that is prominent in a variety of ecosystems in tropical and subtropical regions. Over the past decade, the genome mining of this bacterium has revealed several refined mechanisms related to its remarkable and functional adaptability (BRAZILIAN NATIONAL GENOME PROJECT CONSORTIUM, 2003). Genome annotation analyses of *C. violaceum* identified the existence of open reading frames (ORFs) with high similarity to sequences targeting paraquat-inducible (*pqi*) genes previously characterized in *Escherichia coli* (FARR; KOGAMA, 1991). Notably, it is also well clarified that the *pqi* genes are strongly modulated by various oxidizing agents to minimize their deleterious effects on cellular homeostasis in various bacterial strains (KOH; ROE, 1995; 1996). Moreover, Koh and Roe (1996) reported that the promoter of the *pqiA* gene in *E. coli* is activated by several known superoxide generators, causing a marked induction of expression levels of β-galactosidase in fusion strains. The dual regulation of the *pqi* genes by members of the soxRS regulon suggests indeed that these genes have biological roles in the molecular mechanisms of cellular protection in oxidative stress (KOH; ROE, 1996). Among the broad range of superoxide-generating agents, paraquat is a potent toxic chemical that is widely used in developing countries as an herbicide (plant killer), primarily for weed and grass control. Simply one large oral dose of paraquat is enough to cause burning of the mouth and throat, which is subsequently followed by gastrointestinal tract irritation, abdominal pain, nausea, vomiting, and diarrhea, even leading to convulsions and death due to respiratory failure (GILBERT, 2004).

Plumbagin is one of the simplest plant secondary metabolites of three major phylogenic families Plumbaginaceae, Droseraceae, and Ebenceae, and exhibits highly potent biological activities, including antiinflammatory, anticancer, antibacterial, and antifungal activities (PADHYE et al., 2012). Several studies suggest that these activities arise mainly out of its ability to undergo redox cycling, generating reactive oxygen species and chelating trace metals in biological systems (TILAK et al., 2004; KLAUS et al., 2010). However, some lines of evidence suggest that the toxicity of the plumbagin may not be due to the production of reactive oxygen species, since this compound promotes a loss of galactoside-binding ability and inactivation of NADH-dehydrogenase (IMLAY; FRIDOVICH, 1991; GRANGEIRO et al., 2004; LIN et al., 2010).

Recently, functional properties of regulatory DNA motifs predicted previously in ORFs of *pqi* genes during genome annotation of *C. violaceum* were experimentally elucidate as to the potential activation of these motifs induced by different oxidizing agents (GABRIEL et al., 2015a; 2015b). Because of the importance of experimental approaches for the characterization of regulatory DNA motifs within the bacterial genome, the purpose of this study was to evaluate functionally the effect of plumbagin on the heterologous expression of a predicted promoter region of ORFs of *pqi* genes identified during genome annotation analyses of *C. violaceum*. In addition, the findings described in this study may provide potential biotechnological approaches for the construction of plant transformation

vectors expressing inducible modulated transgenes in a promoter-specific manner in target tissues or in thewhole plant using genetic engineering methods.

Material and Methods

Effect of plumbagin on heterologous expression of *pqi* gene promoter

All experimental steps involved in the amplification and cloning of the amplicon of interest into the conjugative vector were previously described in detail by Gabriel et al. (2015a). A 388-bp amplicon corresponding to the predicted promoter site of the *pqi* gene of *C. violaceum* ATCC 12472 was amplified using specific primers (GABRIEL et al., 2015a) and cloned into the broad-host conjugative vector pMP220, originally carrying the *E. coli lacZ* gene without a promoter and generating transcriptional *lacZ* gene fusions (SPAINK et al., 1987). The *pqi* gene promoter fused to the *E. coli lacZ* gene was electrotransformed into the *E. coli* DH5α strain, as described by Gabriel et al. (2015a). The bacterial inocula carrying the promoter of interest were grown in Luria broth medium (LB, Difco, USA), containing 12.5 µg/mL tetracycline at 30°C for approximately 3 h of incubation under aerobic conditions until they reached an OD_{600nm} of 0.3 by readings in a spectrophotometer (model SL149, Elico Ltd, India). At this moment, bacterial inocula were exposed to 50 µg/mL plumbagin (5-hydroxy-2-methyl-1.4-naphthoquinone, Sigma-Aldrich, USA) in ultrapure water for five hours. The heterologous induction of the predicted promoter region of *pqi* genes of *C. violaceum* was evaluated in the presence of plumbagin by measuring the expression levels of β-galactosidase in the presence of ONPG (ortho-nitrophenyl-β-D-galactopyranoside, *Sigma*-Aldrich, USA), as originally proposed by Miller (1972). Two distinct *E. coli* isolates carrying the promoter of interest fused to the *lacZ* gene, designated clones 12 and 13, were tested under these experimental approaches. Alternatively, *E. coli* cells carrying the above-mentioned *pqi* promoters were also maintained in the absence of plumbagin and served as the control group.

Growth of wild strain of *C. violaceum* in nutrient broth medium in the presence of plumbagin

The growth of a wild strain of *C. violaceum* ATCC 12472 in nutrient broth medium was monitored to examine the existence of an eventual intrinsic resistance of this bacterium in the presence of the oxidizing compound tested. A saturated culture of 100 µL obtained from one isolated colony of *C. violaceum* was grown in selective nutrient broth medium (NB, Difco, USA) in the presence of 250 µg/mL ampicillin. Since *C. violaceum* is ampicillin-resistant (MARTINEZ et al., 2000), this selective antibiotic was used in assays to prevent the growth of other cells that do not possess such resistance. After about 3 h in a shaker incubator (model MA420**,** Marconi Ltda, Brazil) at 30°C under aerobic conditions (250 rpm) until reaching an OD_{600nm} of 0.3, 100 µL of plumbagin in ultrapure water were added to the bacterial inocula at final concentrations of 25, 50 and 60 µg/mL. In addition, 100 µL of ultrapure water were added to a bacterial inoculum, with no added plumbagin. In the control group, there was no addition of ultrapure water or plumbagin to the bacterial inoculum. Bacterial growth was determined by OD_{600nm} measurements at different times of incubation (03:15, 04:15, 05:15, 07:15, 09:15 and 23:59; hours:minutes). All assays were performed in triplicate.

Statistical analyses

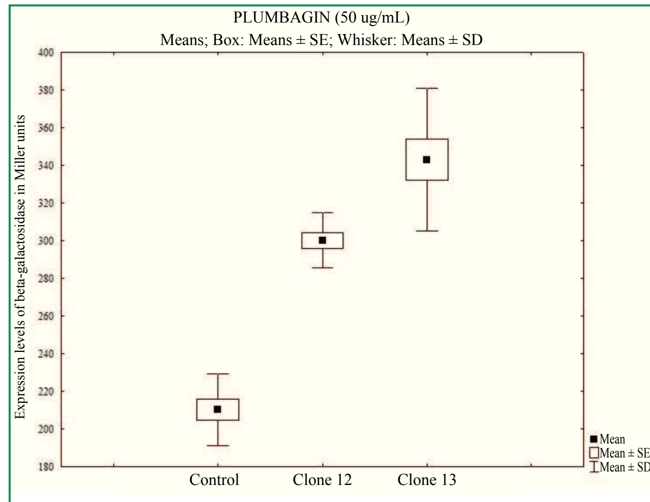
Descriptive statistical analyses of the data were performed using the STATISTICA/W statistical package version 10.0 (Statsoft, USA) from three independent assays. The results presented as means, standard deviations and standard errors of the mean were evaluated using the *hierarchical* linear *model* and analysis of variance with SAS software (SAS INSTITUTE, 1997), where $p<0.05$ was considered statistically significant.

Results

We evaluated the effect of plumbagin on the heterologous expression of a predicted promoter region of the *pqi* genes of *C. violaceum* by measuring

β-galactosidase levels. There was a significant increase in the expression of β-galactosidase modulated by the *pqi* promoter in response to 50 µg/mL plumbagin in comparison to the control group ($p < 0.0001$) (Figure 1).

FIGURE 1: Expression levels of β-galactosidase modulated by a predicted promoter region of the *pqi* genes of *Chromobacterium violaceum* exposed to 50 µg/mL plumbagin. Results are presented as means, standard deviations (SD) and standard errors of the means (SE).

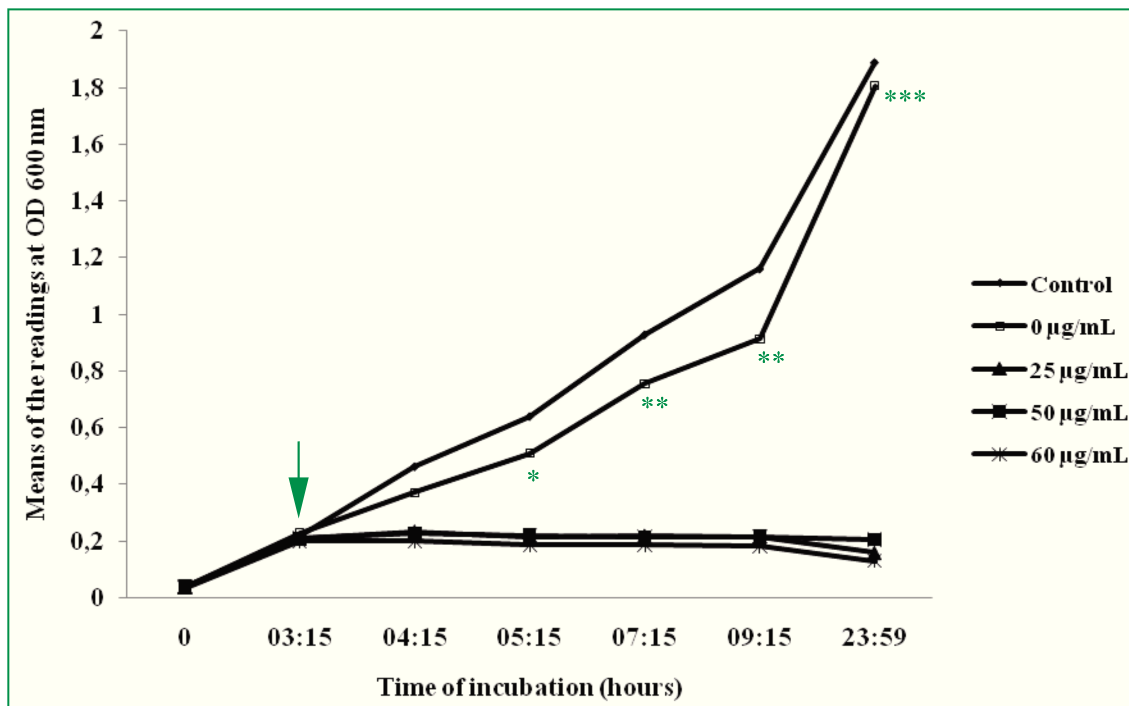


Also, we monitored the growth of a wild stain of *C. violaceum* in nutrient broth medium in the presence of plumbagin at different final concentrations to determine any intrinsic resistance of *C. violaceum* to this oxidizing compound (Figure 2). No differences in bacterial growth were detected up to the addition of plumbagin ($p > 0.005$) (Figure 2). However, irrespective of the plumbagin concentration tested, there was a significantly marked decrease in the growth rate of these bacterial cells with exposure to plumbagin, especially after 2 h of incubation in the presence of this compound ($p < 0.005$) (Figure 2). Bacterial growth of this strain was only detected in the absence of this oxidizing substrate under control conditions (approximately 10^7 colony-forming units per milliliter), as demonstrated in Figure 2.

Discussion

The findings described herein suggest that a potent superoxide radical-generating compound was able to induce a putative promoter region of the *pqi* genes of *C. violaceum* at the concentrations tested. We observed a

FIGURE 2: Growth of wild strain of *Chromobacterium violaceum* in selective nutrient broth medium in response to different concentrations of plumbagin. Incubation times were randomly selected at 03:15, 04:15, 05:15, 07:15, 09:15 and 23:59 hours:minutes. The arrow indicates the moment of the addition of plumbagin. Significant differences compared to control: *p<0.05, **p<0.01, ***p<0.001.



significant increase in the expression of β-galactosidase modulated by a *pqi* promoter region in the presence of 50 µg/mL plumbagin compared to the control group (Figure 1). Differences in β-galactosidase expression levels were detected between clones 12 and 13 (Figure 1), and this variation could be explained by intrinsic fluctuations within each individual clone, such as: randomness in the genetic expression, cell-to-cell variability of the internal biochemical machinery and variation in cell proliferation rate, as previously postulated by Canela-Xandri et al. (2010).

Recent efforts have examined the heterologous activation of regulatory DNA motifs of *C. violaceum* in response to superoxide radical-generating substances. Within this perspective, Gabriel et al. (2015a) reported that paraquat caused significant increases in β-galactosidase expression in an *E. coli* strain carrying the predicted promoter sequence for *pqi* genes of *C. violaceum* fused to the *lacZ* gene. Moreover, Gabriel et al. (2015b) evaluated β-galactosidase expression levels in response to heterologous activation of same predicted promoter region of *pqi* genes in *C. violaceum* induced by menadione (MEN) and phenazine methosulfate (PMS) at different final concentrations and demonstrated that these compounds activated this predicted promoter of *pqi* genes of *C. violaceum* in a dose-dependent manner. Under these experimental conditions, MEN caused highly significant increases in β-galactosidase expression modulated by activating the promoter region of *pqi* genes at all concentrations tested (GABRIEL et al., 2015b). On the other hand, a significantly higher β-galactosidase expression was detected with PMS only at 50 µg/mL (GABRIEL et al., 2015b). These authors concluded that superoxide radical-generating compounds activate a predicted promoter site for paraquat-inducible genes of *C. violaceum* in a dose-dependent manner (GABRIEL et al., 2015b).

Although *C. violaceum* is commonly exposed to variable abiotic conditions, such as changes in temperature and pH, toxic compounds and UV rays (HUNGRIA et al., 2004), a significant decrease in bacterial growth rate was found when exposed to plumbagin at the concentrations tested (Figure 2). Such findings could be explained by the stronger susceptibility of this microorganism to the concentrations of plumbagin selected in this study. Few reports in the literature have described the resistance of this bacterium to toxic chemicals (DAL'MOLIN et al., 2009), and thus, further studies are needed to examine the *in vitro* resistance of the wild strain of *C. violaceum* exposed to plumbagin at other concentrations.

Over the past decade, genomic studies of *C. violaceum* have identified potential mechanisms of pathogenicity (BRITO et al., 2004; CASTRO-GOMES et al., 2014), biotechnological potential (CIPRANDI et al., 2012), and environmental adaptation (SILVA et al., 2004; CORDEIRO et al., 2013). Therefore, this microorganism should be endowed with intrinsic biological features that confer its remarkable competitiveness and ability to survive under different types of environmental stress. Notably, our findings showing the molecular characterization of a promoter sequence of *pqi* genes detected in the *C. violaceum* genome that is sensitive to modulation by a superoxide radical-generating substance seems to support evidence about refined protective systems triggered in this bacterium.

In conclusion, the significant effects of plumbagin on the heterologous induction of a promoter region of the *pqi* genes predicted in the *C. violaceum* genome reinforce the influence of a superoxide radical-generating compound on the activation of a molecular response typically related to great environmental adaptability and refined protective systems inherent to this bacterium.

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