REGULATION OF THE POLYKETIDE PHYTOTOXIN CORONATINE IN *PSEUDOMONAS SYRINGAE*

By

XINWEI WANG

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Peking University

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Dean of the Graduate College

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CHAPTER 1

GENERAL INTRODUCTION

As humans, we are always trying the best to survive a tough life by being stronger, more adaptive and more competitive. So are bacteria. Though not able to think the way we do, they somehow achieve the same goal. Successful plant pathogens invade healthy plants and make them ill. While walking around in the field and seeing those seedlings dying from bacterial diseases, you may feel sorry for their vulnerability. However, usually they are not that vulnerable, only looking so because the pathogens overpowered their defense systems. How pathogens overcome plant defense continues to be an active area of inquiry.

In this project, we deal with two pathogens, one from tomato and the other from soybean, and a chemical named coronatine, which is secreted by both pathogens. Why do the pathogens produce coronatine? What effects does coronatine have on the plants? How can coronatine help the pathogens establish a successful infection? And how do the plants respond to the infection? These are questions that interest us. Although this study does not address all of these questions, it contributes to our overall knowledge of the world of *Pseudomonas syringae*.

CHAPTER 2

LITERATURE REVIEW

Pseudomonas syringae

Pseudomonas syringae, a Gram-negative plant pathogenic bacterium, induces a wide variety of symptoms on plants including blights (rapid death of tissue), leaf spots, and galls (Alfano and Collmer, 1996). The species is subdivided into pathovars (pvs.), or pathogenic variants, according to host range. Two distinct host reactions may be observed when *P. syringae* cells are infiltrated into plant tissue: susceptible host cells develop a water-soaking symptom, followed by pathogen proliferation and advanced symptom development; in contrast, resistant host cells undergo a reaction known as the hypersensitive response (HR), which leads to necrosis in 12-24 h and severely restricts multiplication of the pathogen and thus results in host resistance (Willis *et al.*, 1991). The genetic basis of pathogenicity and virulence in *P. syringae* is complex and includes global regulators (Hrabak and Willis, 1992), the *hrp* cluster, and virulence factors such as phytotoxins and exopolysaccharides (Bender *et al.*, 1999; Yu *et al.*, 1999). The *hrp* region (for *h*ypersensitive *r*esponse and *p*athogenicity) is conserved in phytopathogenic prokaryotes and affects the ability of a bacterium to induce a hypersensitive response

(HR) in nonhost plants, pathogenicity on host plants, and the ability to grow within or on the surface of plants (He, 1998; Hirano *et al.*, 1999). A subset of the *hrp* genes was renamed *hrc* (HR and *c*onserved) because of their conservation in the type III secretion apparatus used by *Yersinia*, *Shigella*, and *Salmonella* (Bogdanove *et al.*, 1996; Galán and Collmer, 1999). The *hrp* genes have been extensively characterized in *P. syringae* where they are clustered in the chromosome and encode regulatory, secretory, or effector proteins (Galán and Collmer, 1999).

Biological effects of coronatine

Many fungal and bacterial pathogens produce secondary metabolites that are toxic to plant cells. Some of these metabolites are phytotoxins, which can directly injure living host protoplasts and influence the course of disease development. Phytotoxins may have the same host-specificity as the producing pathogen, or a wider host range than the producing pathogen. Coronatine (COR) belongs to the latter group. The primary symptom elicited by this non-host-specific toxin is a diffuse chlorosis that can be induced in a variety of plants, including those that cannot be infected by the COR-producing pathogen. COR also induces hypertrophy, inhibits root elongation and stimulates ethylene production (Kenyon and Turner, 1992; Gnanamanickam *et al.*, 1982; Sakai *et al.*, 1979). COR-producing *P. syringae* pathovars include *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato*, which are pathogens of ryegrass, soybean, crucifers, *Prunus* spp., and tomato, respectively. The role of COR varies in different host-pathogen interactions. *P. syringae* pv. *tomato* DC3000 COR^- mutants were found to be

nonpathogenic, indicating that COR is required for the pathogenicity of DC3000. However, COR production is dispensable in some strains of pvs. *glycinea, maculicola, morsprunorum* and *tomato*, which cause plant disease without producing the toxin (Ullrich *et al.*, 1993; Mitchell *et al.*, 1983; Mitchell, 1982). Even in host-pathogen interactions where COR is not required for pathogenicity, COR can significantly increase disease severity. Nontoxigenic strains of *P. syringae* pv. *glycinea* do not induce the stunting, systemic chlorosis, or leaf elongation associated with COR production (Gnanamanickam *et al.*, 1982). Furthermore, the lesions produced by COR-defective mutants of *P. syringae* pv. *tomato* strain PT232 were significantly smaller than the COR-producing wild-type and the population levels achieved by the mutants were much lower than the toxigenic wild-type (Bender *et al.*, 1987).

Structure and biosynthetic route to coronatine

COR has an unusual structure consisting of two parts: coronafacic acid (CFA), which is of polyketide origin, and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Fig. 1) (Ichihara *et al.*, 1977). These two distinct moieties are derived from separate biosynthetic pathways and coupled via amide bond formation (Parry *et al.*, 1994). The biosynthesis of CMA initially involves the isomerization of L-isoleucine to L-alloisoleucine, and the latter is converted by an oxidative cyclization to form the cyclopropane ring of CMA (Fig. 1) (Parry *et al.*, 1994; Parry *et al.*, 1991). The CFA portion of COR is a polyketide derived from three acetate units, one butyrate unit and one pyruvate unit (Fig.1) (Parry *et al.*, 1994). Studies have suggested that the

pyruvate used for CFA biosynthesis is actually converted into α-ketoglutarate before incorporation into CFA, and that α-ketoglutarate may serve as the starter unit for CFA assembly (Parry *et al.*, 1996). Both CFA and CMA are intermediates in the pathway to COR and are secreted by COR-producing strains at low levels (Mitchell *et al.*, 1994; Young *et al.*, 1992). The coupling of CMA and CFA to form COR via amide bond formation is presumed to be the final step in COR biosynthesis (Fig. 1).

Mode of action for coronatine

The exact mode of action for COR, which is still unknown, has been investigated by several researchers by comparing the structural and functional homologies between COR and methyl jasmonate (MeJA), a plant hormone produced in a variety of plants in response to biological stress. Both COR and MeJA induced the accumulation of proteinase inhibitors in tomato leaves and inhibited root growth in *Arabidopsis* seedlings (Palmer and Bender, 1995; Feys *et al.*, 1994). Furthermore, a COR-insensitive mutant of *Arabidopsis thaliana* was also insensitive to the effect of MeJA (Feys *et al.*, 1994). The relatedness between COR and MeJA suggest a similar mode of action, i.e. COR may function as a molecular mimic of the octadecanoid signaling molecules of higher plants (Feys *et al.*, 1994, Koda *et al.*, 1996; Weiler *et al.*, 1994). Although COR, CFA and MeJA all induced proteinase inhibitors in tomato tissues, there were several changes found only in tissue exposed to COR. In COR-treated tomato tissue, chloroplasts were significantly smaller and stained more intensely; the cell wall was thickened and there was visible chlorosis. These phenomena were not observed in tissues treated with CFA or

MeJA (Palmer and Bender, 1995). Consequently, the CMA moiety or the amide bond between CFA and CMA may impart additional biological activities to COR in tomato tissues. Therefore COR does not simply function as a mimic of MeJA in tomato. A receptor for COR needs to be located to precisely clarify the mode of action.

Coronatine gene cluster

The biosynthesis of COR in *P. syringae* pv. *glycinea* PG4180 has been intensely studied because this strain is easy to manipulate genetically, synthesizes large amounts of COR *in vitro,* and infects soybean, a host that is easy to cultivate. By Tn*5* insertion mutagenesis, the COR biosynthesis genes in *P. syringae* pv. *glycinea* PG4180 were located on a 90-kb plasmid designated p4180A (Bender *et al.*, 1991). A functional map of the entire COR cluster has been constructed and a 32-kb region was defined that is absolutely required for COR production (Young *et al.*, 1992; Bender *et al.*, 1993). The COR biosynthetic gene cluster and flanking regions in PG4180 have been completely sequenced (Alarcón *et al.*, 1999; Penfold *et al.*, 1996; Liyanage *et al.*, 1995; Rangaswamy *et al.*, 1998a, b; Ullrich and Bender, 1994; Ullrich *et al.*, 1995). The biosynthetic genes for CMA and CFA are located on opposing ends of the COR gene cluster and are separated by a 3.4-kb regulatory region (Fig. 2B).

COR is the only bacterial phytotoxin known to be under the control of plasmid-borne genes (Coplin 1989; Kinscherf *et al.*, 1991). However, plasmid control of COR synthesis is not a universal rule. Studies have shown that the COR gene cluster spans more than 30 kb of genomic DNA in *P. syringae* pv. *tomato* DC3000 (Moore *et al.*, 1989; Ma *et al.*,

1991). Interestingly, the CFA and CMA genes are physically separated in DC3000 (Alarcón-Chaidez and Bender, unpublished). Futhermore, several strains of *P. syringae* pv. *maculicola* encode the COR gene cluster on chromosomal DNA (Cuppels and Ainsworth, 1995, Zhao *et al.*, 2000). The organization of the COR gene cluster in *P. syringae* pv. *maculicola* is not completely understood.

P. syringae pv. *tomato* DC3000, a pathogen that produces COR and infects tomato, *Brassica* spp. (cabbage, cauliflower), and *Arabidopsis thaliana* (Bent *et al.*, 1992; Moore *et al.*, 1989; Zhao *et al.*, 2000), has become a model strain for investigating plant-microbe interactions, largely because of its genetic tractability and pathogenicity on *Arabidopsis*. As shown in Table 1, PG4180 and DC3000 differ in the amount of COR produced, the genomic location of the COR genes, and the signals for COR gene induction. Furthermore, COR is required for the pathogenicity of DC3000 on tomato and *Arabidopsis* (Mittal and Davis, 1995; Peñaloza-Vázquez *et al.*, 2000). In contrast, COR$^-$ mutants of PG4180 can still initiate disease in soybean, although at a reduced level.

Regulation of coronatine biosynthesis

A variety of nutritional, environmental and host factors have been examined for their effects on COR biosynthesis. COR production in *P. syringae* pv. *glycinea* PG4180 is significantly affected by osmolarity, temperature and carbon source (Palmer and Bender, 1993). COR production in PG4180 was maximal at 18°C and negligible at 28°C (Palmer and Bender, 1993), while the growth of PG4180 was not significantly different at these temperatures. This may explain why the severity of diseases caused by *P. syringae* pv.

glycinea is increased in cool weather. In addition, the biosynthesis of CFA and CMA have the same response to temperature, with optimal production at 18°C. The results obtained by Rohde *et al.* (1998) indicate that COR synthesis is also thermoregulated in *P. syringae* pv. *tomato* DC3000. The optimal temperature for COR production in this strain was 18°C, and no coronatine was detected at 28°C. Furthermore, results obtained with a CMA transcriptional fusion indicated that promoter activity was about four-fold higher when the bacteria were grown at 18°C as compared to 28°C (Rohde *et al.*, 1998).

Sequence analysis of the 3.4-kb COR regulatory region in PG4180 revealed the existence of three regulatory genes, *corP, corS* and *corR* (Ullrich *et al.*, 1995). The predicted protein products of these genes showed homologies to sensor and response regulator proteins in two-component regulatory systems. CorP and CorR, which are transcribed in opposite orientations, showed relatedness to response regulators, whereas CorS is similar to histidine protein kinases that function as environmental sensors. However, the COR regulatory system is modified from the two-component paradigm, because it contains two response regulators together with a single sensor. Mutations in any of these genes abolished the production of CFA and CMA and the temperature-dependent activation of the COR structural gene transcripts (Ullrich *et al.*, 1995; Liyanage *et al.*, 1995). Methods were developed for overproducing the COR regulatory proteins in *P. syringae* (Peñaloza-Vázquez *et al.*, 1996), which made it possible to purify these proteins from their native host and examine their role in signal transduction. Transcriptional fusions, gel retardation assays, and DNaseI footprinting experiments were used to demonstrate that CorR functions as a positive activator of *cor* gene expression and binds to the promoter regions of the *cfl*/CFA and *cmaABT* transcripts (Peñaloza-

Vázquez and Bender, 1998; Wang *et al.*, 1999). CorS was shown to be autophosphorylated by [γ-^{32}P]ATP (forming CorS~P) and has characteristics consistent with phosphorylation at a histidine residue (Rangaswamy and Bender, 2000). Furthermore, the transphosphorylation of CorR by CorS~P was observed within 5 s, suggesting that CorR may be the cognate response regulator for CorS (Rangaswamy and Bender, 2000).

σ^{54} is required for hrp and cor gene expression. σ^{54} (σ^{N}), which is encoded by *rpoN*, is required for a variety of metabolic functions including the utilization of alternative carbon and nitrogen sources, nitrogen fixation, and the expression of virulence determinants (Merrick, 1993). Several reports implicate *rpoN* in the regulation of the *hrp* gene cluster in *P. syringae* (Grimm *et al.*, 1995; Xiao *et al.*, 1994). The *hrpL* gene product, which belongs to the family of extracytoplasmic sigma factors, contains a promoter region with strong homology to the consensus recognized by σ^{54} (Xiao *et al.*, 1994). Recently, σ^{54} was shown to be required for the transcription of *hrpL* in *P. syringae* pv. *maculicola* strain ES4326 (Hendrickson *et al.*, 2000a). The alternative sigma factor encoded by *hrpL* (σ^{L}) controls the expression of several transcripts in the *hrp* gene cluster.

An *rpoN* mutant of *P. syringae* pv. *maculicola* ES4326 was nonpathogenic on *Arabidopsis* and unable to initiate a hypersensitive response in tobacco (Hendrickson *et al.*, 2000b), which is consistent with the involvement of σ^{54} in *hrp* gene expression. Surprisingly, COR production and *cor* gene expression were defective in the ES4326 *rpoN* mutant (Hendrickson *et al.*, 2000b). Although the role of σ^{54} in the synthesis of

COR remains unclear, it is tempting to speculate that σ^{54} may coordinately regulate *hrp* and *cor* gene expression in *P. syringae*.

Cross-talk between the hrp and cor gene clusters. As mentioned above, the type III secretion system encoded by the *hrp* gene cluster is required for pathogenicity in *P. syringae*. The DC3000 Hrp system secretes two classes of proteins that elicit responses in plants: the "harpins" (Charkowski *et al.*, 1998; He *et al.*, 1993); and the Avr proteins that control host range (Mudgett *et al.*, 1999; van Dijk *et al.*, 1999). An intriguing question is whether *hrp* mutants of *P. syringae* would synthesize phytotoxins such as COR when their ability to function as pathogens has been compromised. The availability of defined *hrp* mutants has made it possible to address potential interactions between the *hrp* and *cor* gene clusters in *P. syringae* pv. *tomato* DC3000. Recently, mutations in the *hrp*-encoded type III secretion system were shown to have regulatory effects on COR production in DC3000 (Peñaloza-Vázquez *et al.*, 2000). Although the genetic basis for *hrp-cor* cross-talk is unclear, it should be possible to analyze the regulatory connection between *hrp* and *cor* using molecular approaches.

Induction of *cor* genes *in planta*. Several approaches have been utilized to investigate the potential stimulation of COR synthesis by host plants. Palmer and Bender (1993) amended the growth medium for PG4180 with extracts from soybean tissue or with plant-derived secondary metabolites but found no evidence that these substances substantially increased COR production *in vitro*. In a subsequent study, the activity of transcriptional fusions to the CMA and CFA promoters was compared *in vitro* and in soybean leaves; however, there was no evidence that COR gene expression in PG4180 was higher in plant tissue (Palmer and Bender, unpubl). However, Ma *et al.* showed that COR biosynthesis in

P. syringae pv. *tomato* DC3000 is plant-inducible. Gene fusions indicated that a single transcriptional unit designated CorII was expressed at a higher level *in planta* than *in vitro* (Ma *et al.*, 1991). By inoculating strain DC3000 to several plants, Li *et al.* (1998) found that several hosts had COR-inducing activity, with maximal expression occurring on cabbage. Based on HPLC fractionation and bioassays, they identified tomato leaf factors that activated COR gene expression in this strain and concluded that the active components of crude tomato leaf extract and intercellular fluids were malic and citric acids, with minor contributions from shikimic and quinic acid (Li *et al.*,1998). These observations suggest that there are signals in plants for the induction of COR biosynthesis, and that DC3000 responds to these signals.

COR is required for the pathogenicity of *P. syringae* pv. *tomato* DC3000 in both tomato and *Arabidopsis* (Mittal and Davis, 1995; Peñaloza-Vázquez *et al.*, 2000). Tomato plants sprayed with *P. syringae* pv. *tomato* DC3000 developed typical symptoms of bacterial speck disease, which include necrotic lesions surrounded by the diffuse chlorosis induced by COR (Peñaloza-Vázquez *et al.*, 2000). In contrast, tomato leaves inoculated with the COR$^-$ mutant DC3682 remained symptomless, as did the *hrp* deletion mutant, CUCPB5114 (Peñaloza-Vázquez *et al.*, 2000).

The population dynamics of DC3000, DC3682, and CUCPB5114 were monitored over a 7-day period. The population of DC3000 on day 0 was ~10^5 cfu/ml, and growth of this strain increased steadily and was 1000-fold higher (10^8 cfu/ml) at the end of the sampling period. Interestingly, the COR$^-$ mutant DC3682 did not multiply in the plant, and the population at day 7 was three logs lower than DC3000 and comparable to the *hrp* deletion mutant, CUCPB5114. In subsequent experiments, DC3682 was shown to be

defective in production of CFA, the polyketide component of COR, and could be complemented by cosmid clones containing the CFA gene cluster. The inactivation of the CFA gene cluster in DC3682 eliminated production of COR, CFA, and phytotoxic analogues of COR in the mutant (Peñaloza-Vázquez *et al.*, 2000).

These results agree with a previous report where *Arabidopsis* and tomato plants were inoculated with DC3661, another COR$^-$ mutant of DC3000 (Mittal and Davis, 1995). Symptoms did not develop with DC3661 on either host when plants were inoculated by dipping leaves into bacterial suspensions, and multiplication of the COR$^-$ mutant was several logs lower than DC3000 (Mittal and Davis, 1995). The accumulation of several defense-related genes was higher in *Arabidopsis* leaves infiltrated with the COR$^-$ mutant DC3661 as compared to DC3000, which suggested that COR production by the wild-type DC3000 might actually suppress the defense response (Mittal and Davis, 1995).

CHAPTER 3

OBJECTIVES

The overall objective of this project is to investigate the regulation of COR production in *Pseudomonas syringae* pv. *glycinea* PG4180 and pv. *tomato* DC3000, with special emphasis on regulation *in planta* and *in vitro*. For this work, I focused on the promoter region upstream of the *cfl* gene. In PG4180, the CFA biosynthetic gene cluster consists of ten co-transcribed genes, and *cfl* is located at the 5' end (Fig. 2D). Preliminary work in our lab has suggested that the CFA gene cluster is arranged similarly in DC3000 (Alarcón and Bender, unpubl.). I used transcriptional fusions between the *cfl* gene and a promoterless glucuronidase gene (*uidA*) to investigate differential regulation of COR biosynthesis in PG4180 and DC3000.

CHAPTER 4

DIFFERENTIAL REGULATION OF CORONATINE BIOSYNTHESIS IN *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000 AND *P. SYRINGAE* PV. *GLYCINEA* PG4180

Abstract

Coronatine (COR), a nonhost-specific phytotoxin that significantly increases disease severity, is produced by *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* PG4180. Although the organization of the COR gene cluster differs in these two strains, the structural genes are conserved. In the present study, the *cfl* (*c*orona*f*acate *l*igase) promoter regions from PG4180 and DC3000 were cloned as transcriptional fusions to a promoterless *uidA* gene and used to investigate the regulation of COR production. *In vitro* assays indicated that the *cfl* promoter from both strains was activated very early (6–24 h) in DC3000, with expression decreasing dramatically thereafter. However, in PG4180 expression of both promoters increased slowly beginning at 12 h, and transcriptional activity continued to increase when monitored 48 h after inoculation, suggesting that COR biosynthesis is differentially regulated in PG4180 and DC3000.

Expression of the *cfl* promoter *in planta* was studied by histochemical and fluorometric analysis of GUS activity.

Introduction

COR is a nonhost-specific phytotoxin that is produced by many *P. syringae* pathogens. A diffuse chlorosis is the typical symptom observed in plants infected by COR-producing strains. The structure of COR consists of two parts: coronafacic acid (CFA), originating from the polyketide pathway, and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977). These two parts are coupled via amide bond formation, a reaction catalyzed by coronafacate ligase, the product of the *cfl* gene (Liyanage *et al.*, 1995). The CFA moiety of COR resembles the plant hormone methyl jasmonate (MeJA), a plant-signaling molecule involved in the response of plants to biological stress (Creelman *et al.*, 1992; Farmer and Ryan, 1992; Mueller *et al.*, 1993), suggesting a similar mode of action between these compounds. However, in tomato tissue, COR induced several changes that were not elicited by methyl jasmonate (Palmer and Bender, 1995).

Both *P. syringae* pv. *glycinea* PG4180 (a pathogen of soybean) and pv. *tomato* DC3000 (a pathogen of tomato and crucifers) produce COR *in vitro* and *in planta*. Compared to PG4180, COR production in DC3000 is significantly lower *in vitro* (Peñaloza-Vázquez *et al.*, 2000); however, COR production in DC3000 is plant-inducible (Ma *et al.*, 1991; Li *et al.*, 1998), which has not been demonstrated for PG4180 (Palmer and Bender, 1993). The COR gene cluster is plasmid-encoded in PG4180 and chromosomally-encoded in DC3000 (Bender *et al.*, 1991; Moore *et al.*, 1989). The COR

gene cluster in PG4180 has been extensively mapped and characterized (Alarcón-Chaidez *et al.*, 1999), However, little is known about the organization of the COR genes on the chromosome of DC3000.

DNA sequence analysis of pEC18 (Ma *et al.*, 1991), a cosmid clone containing part of the DC3000 COR gene cluster, indicated that approximately 500 bp of the *cfl* upstream region is homologous in PG4180 and DC3000 (Alarcón-Chaidez and Bender, unpublished). However, the DC3000 *cfl* promoter region lacked a CorR-binding site, which is present in PG4180 (Peñaloza-Vázquez and Bender, 1998). Since CorR was a positive regulator for COR production in PG4180 (Peñaloza-Vázquez and Bender, 1998), this result implies differential regulation of *cfl* transcription in DC3000. In this study, transcriptional fusions containing the PG4180 and DC3000 *cfl* promoter regions were utilized to evaluate how COR production is regulated *in vitro* and *in planta*.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Pseudomonas* strains were routinely cultured on mannitol-glutamate medium (MG) (Keane *et al.*, 1970) at 28°C. Liquid cultures incubated at 21°C were grown in Hoitink-Sinden minimal medium optimized for COR production (HSC) (Palmer and Bender, 1993). *Escherichia coli* DH5α was used as a host in cloning studies and was grown in Terrific Broth at 37°C (Sambrook *et al.*, 1989). Antibiotics were added to media at the following concentrations (μg/ml): ampicillin, 50; chloramphenicol, 25.

Standard DNA procedures

Isolation of plasmid DNA, agarose gel electrophoresis, restriction digests, ligations, purification of DNA fragments from agarose gels, PCR, transformations, and electroporation were conducted using standard techniques (Sambrook *et al.*, 1989). Detection of homologous fragments by Southern blots was carried out using the Genius Labeling and Detection Kit (Boehringer Mannheim). Oligonucleotide synthesis and automated DNA sequencing were provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Selected constructs were transformed into *P. syringae* by electroporation as described previously (Sambrook *et al.*, 1989).

Construction of transcriptional fusions

Using pFEC4 as a template (Fig. 3B and 4), 1.2-kb and 0.56-kb (Fig. 3C) fragments were amplified by PCR using the following two forward primers (F1 and F2) and a single reverse primer (R) (Fig. 4). Each of the primers contains a *Hin*dIII site (marked in bold below):

F1: 5'-GGG**AAGCTT**GACGGTTTGCGGCCCCGG

F2: 5'-GGG**AAGCTT**CAGGTCGTTACGCCTAGG

R: 5'-GGG**AAGCTT**TGGGCGACGACGCTGCGG

Primers F1 and R were used to construct pBG.CFLa, and F2 and R to construct pCFL.560a (Fig. 3C). The PCR products were digested with *Hin*dIII and ligated into pBBR.Gus to create pBG.CFLa and pCFL.560a, respectively (Fig. 3C). pBBR.Gus is a promoter probe vector containing a promoterless *uidA* gene and chloramphenicol resistance (Peñaloza-Vázquez and Bender, 1998). The ligation mixture was transformed

into *E. coli* DH5α, and recombinants were selected on Luria-Bertani (LB) agar supplemented with chloramphenicol and confirmed by plasmid isolation and restriction enzyme digestion. Both pBG.CFLa and pCFL.560a contained the *cfl*::*uidA* fusion in the transcriptionally active orientation (Fig. 3C), which was confirmed by DNA sequencing.

Plant growth and inoculation procedures

Tomato (*Lycopersicum esculentum* cv. Glamour), collard (*Brassica oleracea var. viridis L.* cv. Vates) and soybean seedlings (*Glycine max* cv. Choska) were inoculated in a growth chamber at 24-25°C, 30-40% relative humidity (RH), with a 12 h photoperiod. Four-week-old plants were spray-inoculated with an airbrush (~8 psi) until leaf surfaces were uniformly wet. Inoculum was prepared from bacteria grown on MG agar containing chloramphenicol at 28°C. Surfactant L77 was added to the inoculum at a concentration of 0.2 μl/ml.

Inoculation of tomato and collard seedings: Plants were maintained under high humidity (≥92%) for 24 h before inoculation. DC3000(pBBR.Gus) and DC3000(pCFLP) were incubated for 36 h on MG agar supplemented with chloramphenicol at 28°C; cells were then suspended ($O.D._{600}$=0.3) in distilled water. After inoculation, tomato and collard plants were incubated at 24°C, with 88% RH. Two humidifiers were placed inside the growth chamber to maintain high RH, which was very helpful for successful inoculation.

Inoculation of soybean seedlings: PG4180.N9(pBBR.Gus) and PG4180.N9(pCFLP) were incubated for 48 h at 28°C on MG agar containing chloramphenicol and then suspended in sterile distilled water at $O.D._{600}$ = 0.4. The inoculum for soybean was higher

than for tomato and collard because more inoculum was required for a successful infection. Soybean plants were incubated in a dew chamber for 2 days before and after inoculation, then transferred to a growth chamber maintained as described above for tomato and collard plants.

Glucuronidase assays

In vitro: Each strain carrying an individual construct was inoculated to triplicate tubes containing 10 ml of HSC and incubated at 21°C. At different time points (0, 6, 12, 24, and 48 h after inoculation), bacterial cells were removed from each tube for GUS assays as described previously (Peñaloza-Vázquez and Bender, 1998). The protein concentration of cell lysates was determined using the Bio-Rad (Richmond, Calif.) protein assay kit following instructions provided by the manufacture. Glucuronidase activity (GUS) was expressed in units (U) per mg of protein, with 1 U equivalent to 1 nmol of methylumbelliferone formed per min. The GUS activities of strains containing the vector pBBR.Gus were included as a control.

In planta: Plants were spray-inoculated as described above. At different time points (0, 12, 24, 48, 96 and 168 h after inoculation for tomato and collard; 0, 48, 96, 168, and 240 h after inoculation for soybean), three leaves from three replicate plants were removed, weighed and macerated in 2 ml sterile water. From the homogenate of each leaf, 10 µl was used for bacterial counts and plated on MG agar with chloramphenicol (25 µg/ml). Another 500 µl was mixed with 2x GUS extraction buffer and kept at -80°C for future GUS activity assays (Peñaloza-Vázquez and Bender, 1998).

Histochemical detection of glucuronidase activity

Tomato, collard and soybean leaves were sampled as described above, and vacuum-infiltrated with a substrate-surfactant solution (5-bromo-4-chloro-3-indolyl β-D-glucuronide, 0.5 mg/ml, and L77 at 0.2 µl/ml in 0.5 mM sodium phosphate buffer, pH 7.0). Infiltrated leaves were incubated at 37°C overnight, and fixed and decolorized in 80% ethanol at 37°C (Hugouvieux *et al.*, 1998).

Results

Sequence analysis and construction of transcriptional fusions

Ma *et al.* (1991) isolated pEC18, a cosmid clone from a genomic library of DC3000 that contained some of the genes involved in COR production. The *cor* genes present in the 30 kb insert in pEC18 were later shown to overlap with the PG4180 COR gene cluster (Cuppels and Ainworth, 1995). This indicated that we could use the COR genes in PG4180 to detect and locate *cor* genes in pEC18. To determine which *cor* genes were present in pEC18, five pairs of primers were designed with each pair corresponding to one region of the PG4180 COR gene cluster: *cfl*, *corR*, *corS*, *cmaA-cmaB*, and *cmaT-cmaU*. Using pEC18 as a template, PCR products were obtained with primers derived from the *cfl* and *cmaA* genes (data not shown). Very faint bands were observed with the other primer sets, suggesting that the corresponding genes were not present or have diverged from PG4180. To further investigate what *cor* genes were present on pEC18, high stringency Southern blotting was utilized. Two clones containing the *cfl* and *cmaA* genes from PG4180 were used to probe the *Sst*I and *Xho*I fragments of pEC18 (Fig. 3A).

Very strong signals were obtained with the *cfl* gene while no homology was observed for *cmaA*, suggesting that the latter gene is not present in pEC18. The *cfl* probe hybridized to a 2.9-kb *Sst*I fragment and an 11.5-kb *Xho*I fragment (Fig. 3A). The 2.9-kb *Sst*I fragment was cloned into pBluescript#II SK+ resulting in pFEC4 (Fig. 3B). Sequence analyses of pFEC4 indicated that the *cfl* promoter in DC3000 was different from PG4180.

The DC3000 *cfl* promoter region lacked a CorR-binding site, which is present in PG4180, implying differential regulation of *cfl* trancription in DC3000. Using the sequence information (Fig. 4), 1.2-kb and 0.56-kb fragments from the *cfl* DC3000 upstream region were cloned into the promoter probe vector, pBBR.Gus, resulting in pBG.CFLa and pCFL.560a, respectively (Fig. 3C). Starting from the 5' end, the first 640 bp of the 1.2 kb fragment in pBG.CFLa contains DNA that is diverged from PG4180 while the rest of it conserved. The 0.56-kb fragment in pCFL.560a contains only DNA sequence that is conserved in both DC3000 and PG4180. These two constructs, together with transcriptional fusion pCFLP (Table 2), which contains the PG4180 *cfl* promoter, were mobilized to PG4180.N9 and DC3000 and transcriptional activity was monitored *in vitro* and *in planta*.

GUS assays *in vitro*

The GUS activity of DC3000 containing pBG.CFLa or pCFL.560a was measured at 0, 12, 24, 36, and 48 h after inoculation to HSC broth. Interestingly, pCFL.560a exhibited a much higher GUS activity than pBG.CFLa (data not shown). Although the reason for this difference is unclear, there could be two possibilities: 1) pBG.CFLa, which contains the larger fragment from the DC3000 *cfl* upstream region, may contain a repressor binding

site; or 2) the secondary structure present in pBG.CFLa but not in pCFL.560a may affect activation of the *cfl* promoter. In all subsequent studies, pCFL.560a was chosen for studying the DC3000 *cfl* promoter.

The transcriptional activity of pCFLP and pCFL.560a was evaluated by measuring GUS activity of DC3000 and PG4180.N9 containing these constructs. PG4180.N9 is derived from the wild-type PG4180, and contains a Tn*5* insertion that does not affect growth or COR production (Ullrich *et al.*, 1994). PG4180.N9 was used in the present study because it is easier to manipulate genetically than PG4180. Interestingly, expression of the two promoters was strain-dependent (Fig. 5A, B). In DC3000, both transcriptional fusions were activated very early after inoculation with transcriptional activity decreasing rapidly afterwards (Fig. 5A, B). In contrast, the expression of both fusions in PG4180 increased slowly beginning 12 h after inoculation (Fig. 5A, B). It is also important to note that the expression level of the *cfl* promoters was different. The GUS activity levels of pCFLP varied from a maximum of approximately 2000 in DC3000 to 9000 U in PG4180 (Fig. 5A). Although no distinctive difference was observed in the expression level of pCFL.560a in DC3000 and PG4180.N9 (Fig. 5A), the overall transcriptional activity of pCFL.560a was significantly lower compared to that of pCFLP (Fig. 5A, B). These results indicate that the *cfl* promoters themselves also impact the expression level, suggesting a strain and promoter effect on the regulation of COR production in PG4180 and DC3000.

Symptom development in inoculated plants

DC3000(pBBR.Gus) and DC3000(pCFLP) were inoculated to tomato and collard seedings, whereas PG4180.N9(pBBR.Gus) and PG4180.N9(pCFLP) were inoculated to

soybean seedings as described above. In tomato, small, dark green water-soaked lesions were observed 48 h after inoculation and developed into necrotic lesions 96 h after inoculation. At 96 h, the chlorosis associated with COR production was apparent around the lesions and finally attained a high level at 168 h (7 days) (Fig. 6A). A similar pattern of symptom development was observed in collard, except that necrotic lesions appeared earlier (72 h after inoculation, Fig. 7A). In soybean leaves inoculated with PG4180.N9 (Fig. 8A), no symptoms were observed until 96 h after inoculation when chlorotic lesions were apparent (Fig. 8A). Necrosis was observed 168 h after inoculation, and necrotic lesions were located inside chlorotic halos (Fig. 8A).

Detection of PG4180 *cfl* promoter activity *in planta*

Histochemical staining was used to further study the transcriptional expression of the PG4180 *cfl* promoter *in planta*. Conceptually, if the *cfl* promoter in pCFLP is activated *in planta*, β-glucuronidase, the product of *uidA* gene, will be produced. When leaves infected with *P. syringae* strains carrying pCFLP are stained with the chromogenic substrate X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), a visible blue precipitate will be formed where the bacteria are located.

Leaves from different plants were sampled at 0, 12, 24, 48, 96, 168 and 240 h after inoculation and stained for GUS expression. On tomato leaves (Fig. 6B), small blue spots were seen as early as 6 h after inoculation, indicating that the *cfl* promoter was activated very soon after DC3000 interacted with tomato. The highest GUS activity in tomato was observed 48 h after inoculation (Fig. 6B). The behavior of the *cfl* promoter in collard (Fig. 7B) was similar to tomato (Fig. 6A). The *cfl* promoter was also activated soon

(within 24 h) after DC3000 was inoculated to collard and the maximal activity observed 72 h after inoculation (Fig. 7B). In contrast, the transcriptional activity of pCFLP in soybean was synchronized with the development of chlorosis. Blue precipitate did not appear until 48 h after inoculation and intensified thereafter, attaining a high level at 240 h (10 days) after inoculation (Fig. 8B).

Quantitative evaluation of GUS activity was conducted using tomato and collard as hosts (Fig. 9A, B). This method was similar to GUS assays *in vitro*, except that GUS activity was determined by measuring the fluorescence of the leaf tissue homogenate. Consistent with histochemical staining, the *cfl* promoter was activated within 24 h and the activity increased rapidly to a high level 48 h after inoculation. Interestingly, GUS activity in collard was higher than tomato, implying a possible host effect.

Discussion

Comparison of the *cfl* promoter regions of DC3000 and PG4180

DNA sequence analysis indicated that the *cfl* promoter regions were different in DC3000 and PG4180, although there were 496 bp conserved immediately upstream of the start codon. The PG4180 *cfl* promoter region contains a CorR-binding site (Peñaloza-Vázquez and Bender, 1998), which is absent in the *cfl* promoter of DC3000. CorR is a regulatory protein that functions as a positive regulator for the transcriptional activation of the *cfl* promoter in PG4180 (Peñaloza-Vázquez and Bender, 1998). In PG4180, CorR was phosphorylated by the histidine protein kinase, CorS, and physically bound the *cfl* promoter in gel shift and DNase I footprinting assays (Peñaloza-Vázquez and Bender,

1998; Ranagaswamy and Bender, 2000). Furthermore, *corR* mutants of PG4180 were defective in the synthesis of COR, CFA, and CMA (Ullrich *et al.*, 1995). Although DC3000 contains a *corR* gene (Alarcón-Chaidez and Bender, unpubl.), the *cfl* promoter of DC3000 lacks a CorR-binding site, implying differential regulation of COR production at the *cfl* promoter in DC3000 and PG4180.

cfl promoter activity in DC3000 and PG4180

Many factors, including osmolarity, temperature and carbon source, affect COR production in PG4180 (Palmer and Bender, 1993). The *in vitro* studies of *cfl* promoter activity in DC3000 and PG4180 indicate that *cfl* expression was strain-dependent. In other words, the expression level of the *cfl* promoter was dependent on the genetic background of the host strain. This was true for both the level of transcriptional activity and the kinetics of *cfl* expression. When monitored *in vitro*, transcriptional activity was much lower in DC3000 when compared to PG4180 (Fig. 5A, B). This result correlates well with the amount of COR synthesized *in vitro* by these strains; DC3000 produces 25 to 40-fold less COR than PG4180 (Table 1). Furthermore, both pCFLP and pCFL.560a (the *cfl* promoters from PG4180 and DC3000, respectively) were activated very early in DC3000, whereas the activity of these two promoters gradually increased with time in PG4180 (Fig. 5A, B). When transcriptional activity was monitored in DC3000(pCFLP) and PG4180(pCFLP) *in planta*, a similar pattern was observed. For example, GUS activity was evident very soon after inoculation when the host strain was DC3000; in contrast, GUS expression increased much later for PG4180 (Fig. 6B, 7B, 8B).

These results have greater implications when considered with respect to the importance of COR in different host-pathogen interactions. For example, COR$^-$ mutants of DC3000 failed to multiply and cause disease in tomatoes (Mittal and Davis, 1995; Peñaloza-Vázquez *et al.*, 2000), indicating that COR is required for symptom development and disease in tomato. Furthermore, COR production by DC3000 was shown to decrease the expression of several genes involved in the plant defense response. Many defense systems of plants are activated very quickly after pathogen attack. Therefore, if COR is to be effective in suppressing such defense systems, it must be expressed very quickly after infection. In the present study, the *cfl* promoter in DC3000 was activated very quickly, which is consistent with a potential role for COR in suppressing host defense.

In contrast, COR$^-$ mutants of PG4180 still cause some disease, although the severity is greatly reduced in comparison with the wild-type PG4180 (Budde and Ullrich, 2000). CFA, the polyketide component of COR, was shown to delay the expression of a hypersenstive response (a defense mechanism) in tobacco, which suggests that COR and CFA may delay the manifestation of defense in other plants (Budde and Ullrich, 2000). Furthermore, *cfl* expression in PG4180 occurs later than in DC3000, and this delay may provide more time for the pathogen to become established and less time for the plant to mount an effective defense response, which possibly explains why COR$^-$ mutants of PG4180 still cause disease in soybean. Thus, the timing of *cfl* gene expression and COR synthesis may play a vital role in the outcome of the host-pathogen interaction.

<u>COR may be required for symptom development in collard and tomato</u>

The *cfl* promoter was activated very early in tomato and collard (Figs. 6B, 7B), which are natural hosts of DC3000. Intriguingly, there was a significant difference in the expression level of the same *cfl* promoter in tomato and collard. In collard, the maximal level of promoter activity was 10-fold higher than in tomato (Fig. 9A, B), indicating that *cfl* gene expression in DC3000 can vary with the host plant. In addition, COR production in DC3000 is plant-inducible (Li *et al.*, 1998); consequently, COR production may be induced to different levels depending on the host plant. When compared to tomato and collard, the *cfl* promoter in PG4180 was activated much later and symptoms developed more slowly, implying that COR production in PG4180 is not required for PG4180, which is consistent with previous results (Palmer and Bender, unpubl).

Table 1

COR Production in *P. syringae* pv. *glycinea* PG4180 and *P. syringae* pv. *tomato* DC3000

	PG4180	DC3000
Production *in vitro*	40-50 μg COR/mg protein	1-2 μg COR/mg protein
Location of COR genes	Plasmid-borne	Chromosomal
Signals for induction	Temperature	Temperature Host
Involvement in pathogenicity	Dispensable, but increases virulence	Required

Table 2

Bacterial Strains and Plasmids

Strains and Plasmids	Relevant Characteristics	References
E. coli JM109		Yanisch-Perron *et al.*, 1985
P. syringae pv. *glycinea* PG4180.N9	COR^+; Km^R	Ullrich *et al.*, 1994
P. syringae pv. *tomato* DC3000	COR^+; Rif^R	Cuppels, D. A.
Plasmids		
pBBR.Gus	Cm^R; 6.6-kb promoter probe vector containing the promoterless *uidA* gene	Peñaloza-Vázquez and Bender, 1998
pBG.CFLa	Cm^R; constains a 1.2-kb fragment from the 5' end of the DC3000 *cfl* upstream region in pBBR.Gus	This study
pCFL.560a	Cm^R; contains a 0.56-kb fragment from the 5' end of the DC3000 *cfl* upstream region in pBBR.Gus	This study
pCFLP	Cm^R; contains a *Pst*I-*Sal*I fragment from PG4180 *cfl* promoter	Peñaloza-Vázquez and Bender, 1998

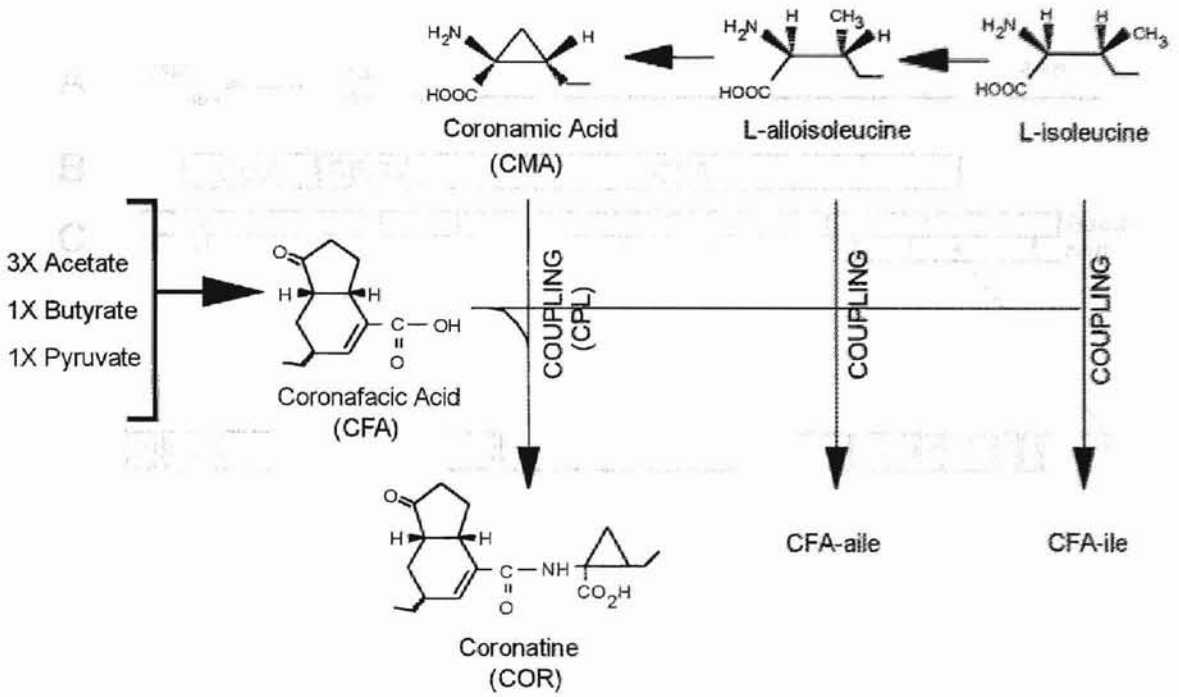


Fig. 1 Biochemical pathways involved in the synthesis of coronatine (COR). COR consists of a polyketide component coronafacic acid (CFA) that is coupled (CPL) via amide bond formation to coronamic acid (CMA). CFA can also be coupled to L-alloisoleucine and L-isoleucine to form the COR analogues, CFA-aile and CFA-ile, respectively.

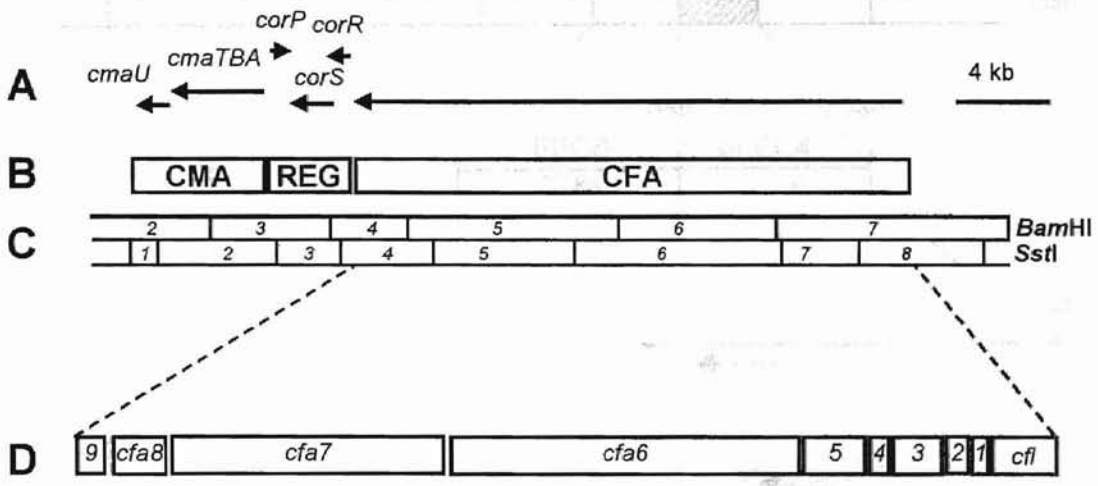


Fig. 2 Functional and physical map of the COR biosynthetic gene cluster in PG4180. (**A**) Horizontal lines with arrowheads indicate the transcriptional organization of the COR gene cluster. (**B**) Functional regions of the COR biosynthetic gene cluster: CMA, CMA biosynthetic gene cluster; REG, regulatory region; and CFA, CFA biosynthetic gene cluster. (**C**) Physical map of the COR gene cluster; enzymes used for restriction mapping were *Sst*I and *Bam*HI. (**D**) Expended view of *Sst*I fragments # 1 to # 8, which contain the CFA biosynthetic gene cluster. Abbreviations: 1, *cfa1*; 2, *cfa2*; 3, *cfa3*; 4, *cfa4*; and 9, *cfa9*.

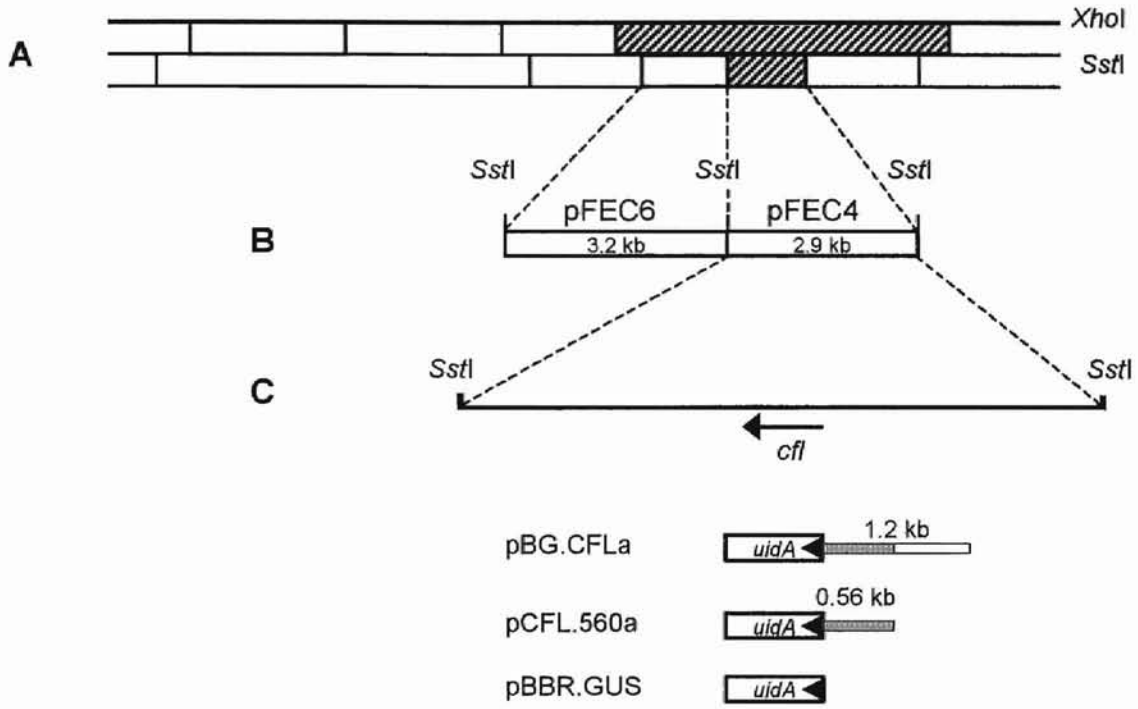


Fig. 3 Location and orientation of the promoter probe constructs used in this study. (**A**) Physical map of the 30-kb fragment of DC3000 genomic DNA on pEC18. Shaded fragments hybridized with the PG4180 *cfl* probe. (**B**) Two *Sst*I fragments, one 3.2 kb and the other 2.9 kb, were used to construct pFEC6 and pFEC4, respectively. (**C**) The location and orientation of the promoter probe constructs used in this study. The conserved DNA region is shaded in gray.

Fig. 4 DNA sequence of pFEC4, a pBluescript clone that contains a 2.9-kb *Sst*I restriction fragment from pEC18, a cosmid clone carrying genes involved in COR production from a genomic library of DC3000. The *Sst*I restriction sites are shaded in gray (▮). Nucleotide sequences used for subcloning by PCR are underlined. The reverse primer R and one of the two forward primers, F1, were used to construct pBG.CFLa, and R and F2 were used to construct pCFL.560a. The sequence conserved in PG4180 *cfl* promoter region is in **bold**.

5' GAGCTCGATACTCTGAAGCACGACCAACGCCGAGCTTTCGAGCAATTTCTGTGGCACCTAGATTTTCTTC

F1 →

71 GGTCCATAAGCGCTGAACTTACAGCGGGTCAATCGACGGTTTGCGGCCCCGGTAAAAACACCACGAGCTT

141 TAGCGTCAGCAATTCCTTCCAGCTGCCGATCGCGTCGCAGGTGAGTTTCAAAATCAGCAAACATGCCCAG

211 CATGTTCAAAAATGCTTTTCCGGCTGCGCTCTGCGTATCGATTGGTTGCTCCGTCCTCTTGAGACTAACC

281 CCACGTTCTTTGAATGTATAAACGATGTCTTGAAGATCCTTGATGCTCCGCGCGAGACGATCAACACGCG

351 TNACCACTGACGTATCGCCAAGGCGTAGAAAATCGAGCAGTGGTTTCAAAATCCGTCCGACCAACCCGGC

421 TAGCCCCGGCTTTTTTCTCGGCGCGCGCGACATCATAACCCGCTTCACGCAAAGTTTTTCTTGCAGAACG

491 AAATCTTAATCGCTTGTGGAAACACGAGTGTATCCGTAAACGGCCATCGTCAAATTTGTCTCTTTAAGCT

561 CTAGAAGGCGAGTGGTCACTCGCAACTAATTCTGGTTCATGCCGGTCGCTAGCAGGTCTAACCAGATGAG

631 TGCAGGTTCATTCGCAAATAATCCCGGTTTGCCGGATCGGATAGACGCAATCAATCCCGGTCGAAAAACG

F2 → Conserved →

701 CGATGATTGCAGGTCGAGGCCATTCAGATTGCAGGTCGTTACGCCTAGGGAGCTTTTGGTCAAAATGGAC

771 **GATGGGCGTTAGCCCATTATGCCTTTAACGTAGGTTCGTGGTTGAGCGTGGGCTATCTCCCTGAACGAGC**

841 **AGATGGTTTGCGTCCCGATGATAATACTTTGGCGAAGGTTACCCTCATTGCGATCAGTCATTCAGCGTTA**

911 **CAGAACCGTTCTAGAAAACGGCTTTGCAGGTGCCTTACGCCAAGCGGACTTAATCCACTCCCCCTTTCCA**

981 **ATGGGGCTTCAGACTTGTCCGCCTCTGCTCCTTGACCGGGCAGCATCAATGAAGCCGACGCTTCCGCAGC**

1051 **AGCGCTTTATGCTCCGACACCATGCGCTGCTCGCGACTGTGGGCCCTCCCAAAACCAAGCATTAGCGGAC**

1121 **CGGTACGTCTGTAAGACGGCCTCCCGTACGGAGGCCCTTGAATCCGGTCCTGCTTAGGGACGCAGCTTCA**

← Conserved *cfl* →

1191 **CAACACGCCTCGTATTTTCCGATCTGCACGCAGGTGCAAAAAAAGGTGATCTAGCATGAGTCTGATTTCT**

← R

1261 GAGTTCCGCAGCGTCGTCGCCCAGCAGCCGGATACCACGGCCGTAGTGGAAGATCAGCGCGCGGTTTCCT

1331 TCACAGAGCTGGCACAACTGGCCGACAAAGTCTCCGCTGGGCTGTTGCAAGCGGGCCTGCAGCCGGGCGA

1401 TCGCGTGGCTATTCACCTAGGTAATCGGCTGGAACTGGTGGCGTTGTACTATGCCTGCCTGGAAATCGGC

1471 GCAGTGACAGTGCCTATCAATCGGCGCCTCGTCACCGGCGAAATTGAGCATCTGCTCCACCACAGCGGCG

1541 CCCGCTACTACATCGGCGACCAGGAGACCTACAGCCGTTACGCCGCTGTGATCGCGGGTAGTGCCACGGT

1611 GGAACGGGCCTGGATCGTCGCGGGCGAGGAAGCGCTCAAGGAGGAGCAGTATCTGGCCTGGTCCGATCTG

1681 CTCGTCTCGTCGCCAAGTAAGCGGCCGCCTAGCCACGCGGATTCGCTCGCGGCGATTTTCTATACGTCCG

1751 GCACCACAGGACTAACTAAGGGTATCGTGCATTCCCAAGCGACCCTGGCGCAGGCCGTCGATCTGATGAA

1821 GGCTATGATGCCACCCCGCACGGCCCAAGGTGCCCTCGACACGGGGGCCGTCCATTCGATGAGGATGCGA

1891 TCGTGCTTGGAAGCATCCTGATGATCCTGCCCGCGCCCGTNTGGCCGGCCCGTCGTGTTGCTACCGGGAC

1961 TTACGGCGAAGACGACCCTGGCGTTATTACAACGACTGCCGNTAGTTTTCTCAAAGGCGTTCCNTGGCAC

2031 TCCAATAATCTGCTCGCTGCAGCCAAGGCATCGGCCAGCCACCCCCCTTCCCTCTCTGACGAGCACCTAT

2091 AGCGTCAGCGCCGGTGACCCCTGCCCTCCGAAGCTGGGTAGGCGTTGGCACAACCTATGGGNCGGCACGT

2161 TGCGTGGTTCCTACGGTACGACGGAGTCTGGCCCGATCTTCTGCCAACCCGATGTAGCCGCCACCGAGCA

2231 GTCATCGATCGGCTGGCCGCTGCCTGGCGTCGCGCTGCAGCAGACGGAGAACGGCGAGCTGTTGATTCGC

2301 TCGCCAGCCAATACCCCGGGACTGTGGAACGGCCAGGATGCAGATCGCCTGCCTGCTGCGCGCTGGATAG

2371 CTACGGGCGACCTGGTGCAGCGCCAGGACGATGGCGGTTATCTCATCATCGGTCGGGAAAAGGACATGCT

2441 GAAATGCGACGCTTACTCCATCTCCCCCGTGGAAGTCGAGCAGGAGCTGCTCAAGCTGCCCGACATCGCC

2511 GAAGCCGTGGTGTTCGGTGTTCCCTGAATGCCCACCATCGGCAAGCGCCCAGTCGCNTNTGTTGCGTACN

2581 ACCCAGTGGCCGGNAAGCTTCCCACGCAACAGNTGAAGCAGCACTTGAAGGCGTTGATCCCAGAATACAA

2651 GCACCCCCGCCAATACCTGTTCGTCGAACGTATTCCGTTGTCCTCCGCGGGNAAGGTGANCCGTAAACAA

2721 TTGGCAAGCGACTACCGAGAGATCCTCGGCGCCGCCCCATCGACCTAGCCCCGTCAGTGACGCGCGTTGC

2791 CTGCGGGACCGGCTCTACAGCCGGCCCCGATACCAACCCTTTGCCCACCACTGGAGCTC 3'

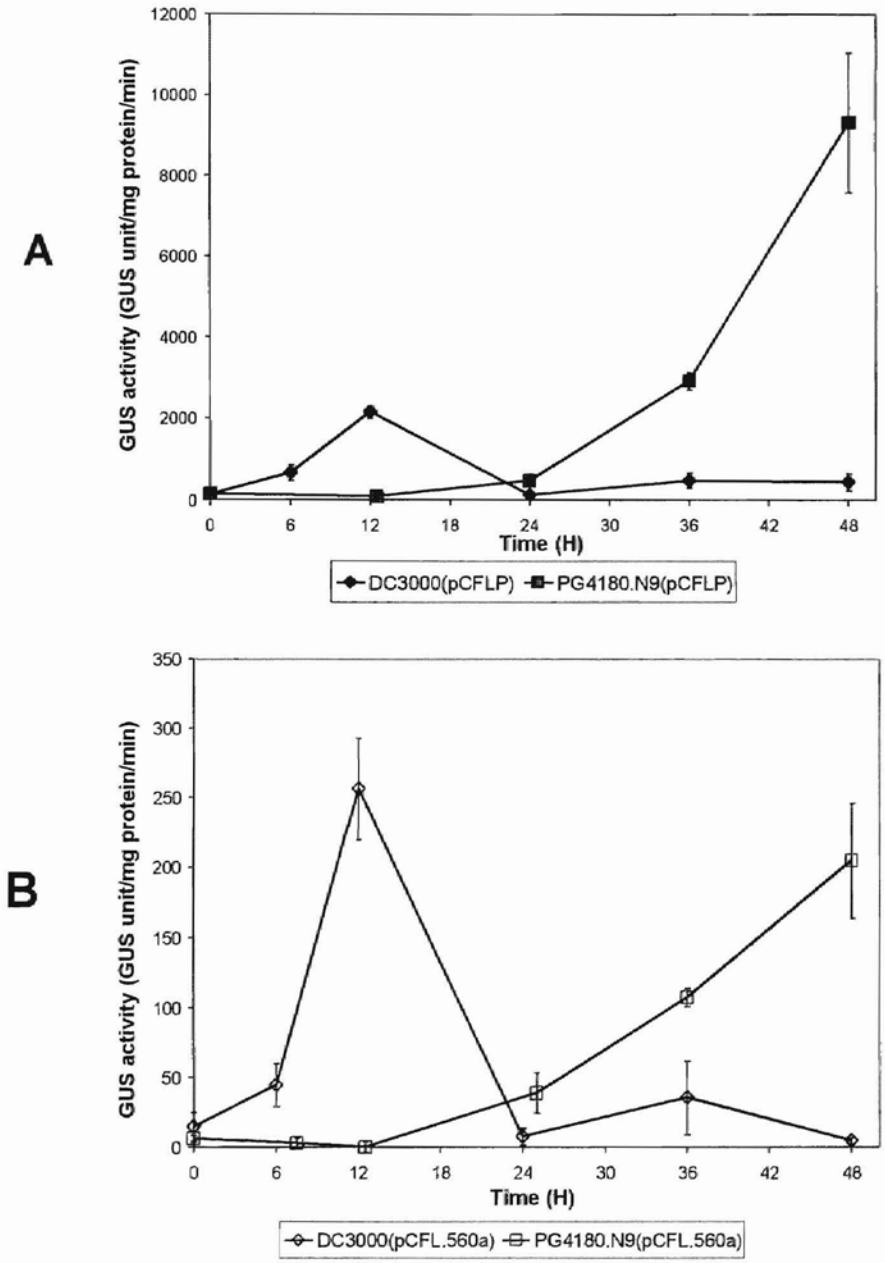


Fig. 5 GUS assays *in vitro* of pCFLP (**A**) and pCFL.560a (**B**) in DC3000 and PG4180.N9 at 21°C. The promoter activity of all the constructs was measured at 0, 6, 12, 24, 36, and 48 h. GUS activity was normalized as a function of protein and presented as GUS unit/mg protein/min. pBBR.Gus was used as a control.

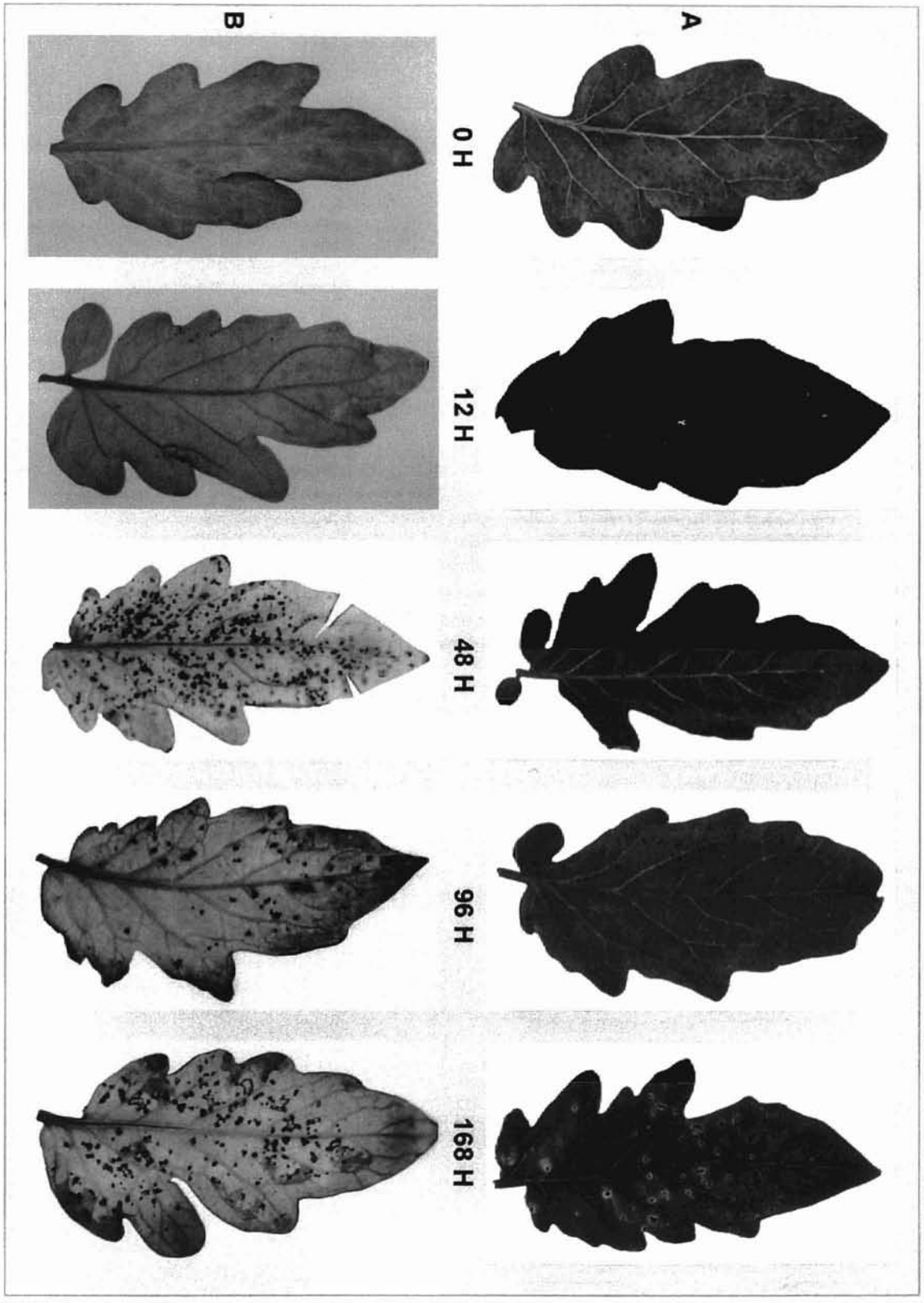

A
B
0 H
12 H
48 H
96 H
168 H

Fig. 6 Symptoms produced by DC3000(pCFLP) and histochemical staining for GUS activity in tomato. (**A**) Symptoms produced by DC3000(pCFLP) at 0, 12, 48, 96, and 168 h after inoculation in tomato. (**B**) Histochemical staining for GUS activity of DC3000(pCFLP) at different time points.

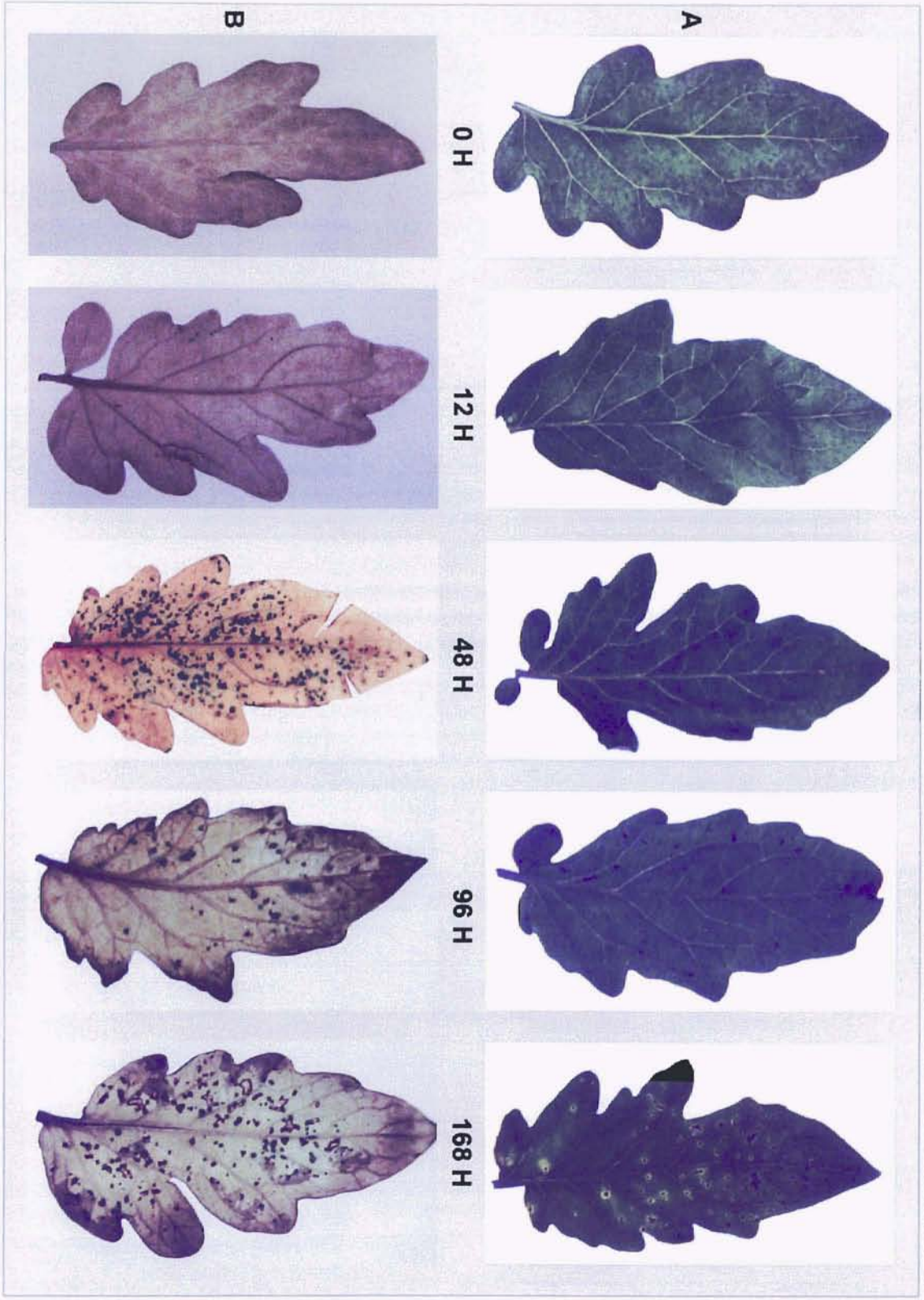

A
B
0 H
12 H
48 H
96 H
168 H

Fig. 7 Symptoms produced by DC3000(pCFLP) and histochemical staining for GUS activity in collard.(**A**) Symptoms produced by DC3000(pCFLP) at 24, 72, 120 and 168 h after inoculation in collard. (**B**) Histochemical staining for GUS activity of DC3000(pCFLP) at different time points.

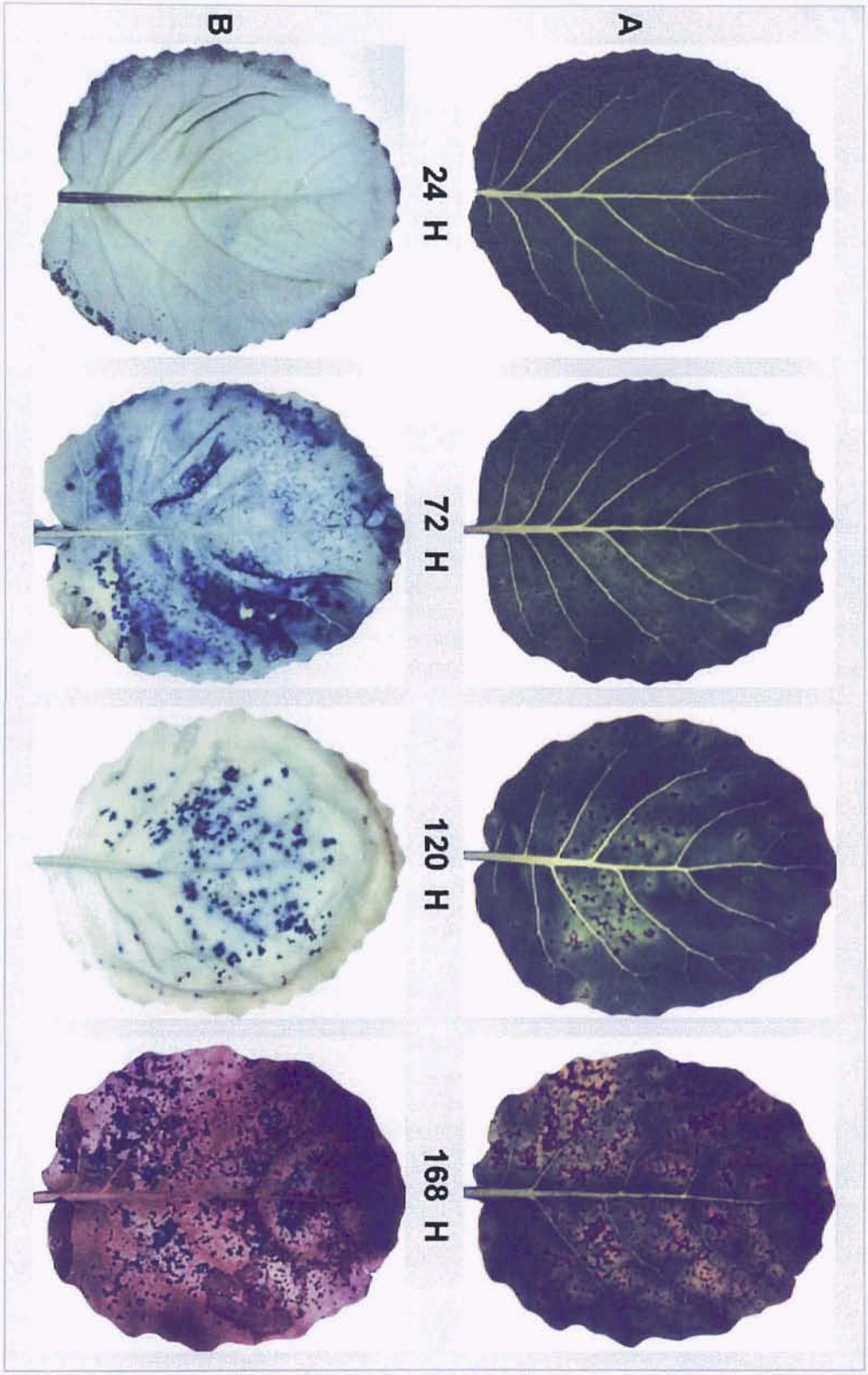

A
B
24 H
72 H
120 H
168 H

Fig. 8 Symptoms produced by PG4180.N9(pCFLP) and histochemical staining for GUS activity in soybean. (**A**) Symptoms produced by PG4180.N9(pCFLP) at 0, 48, 96, 168, and 240 h after inoculation in soybean. (**B**) Histochemical staining for GUS activity of PG4180.N9(pCFLP) at different time points.

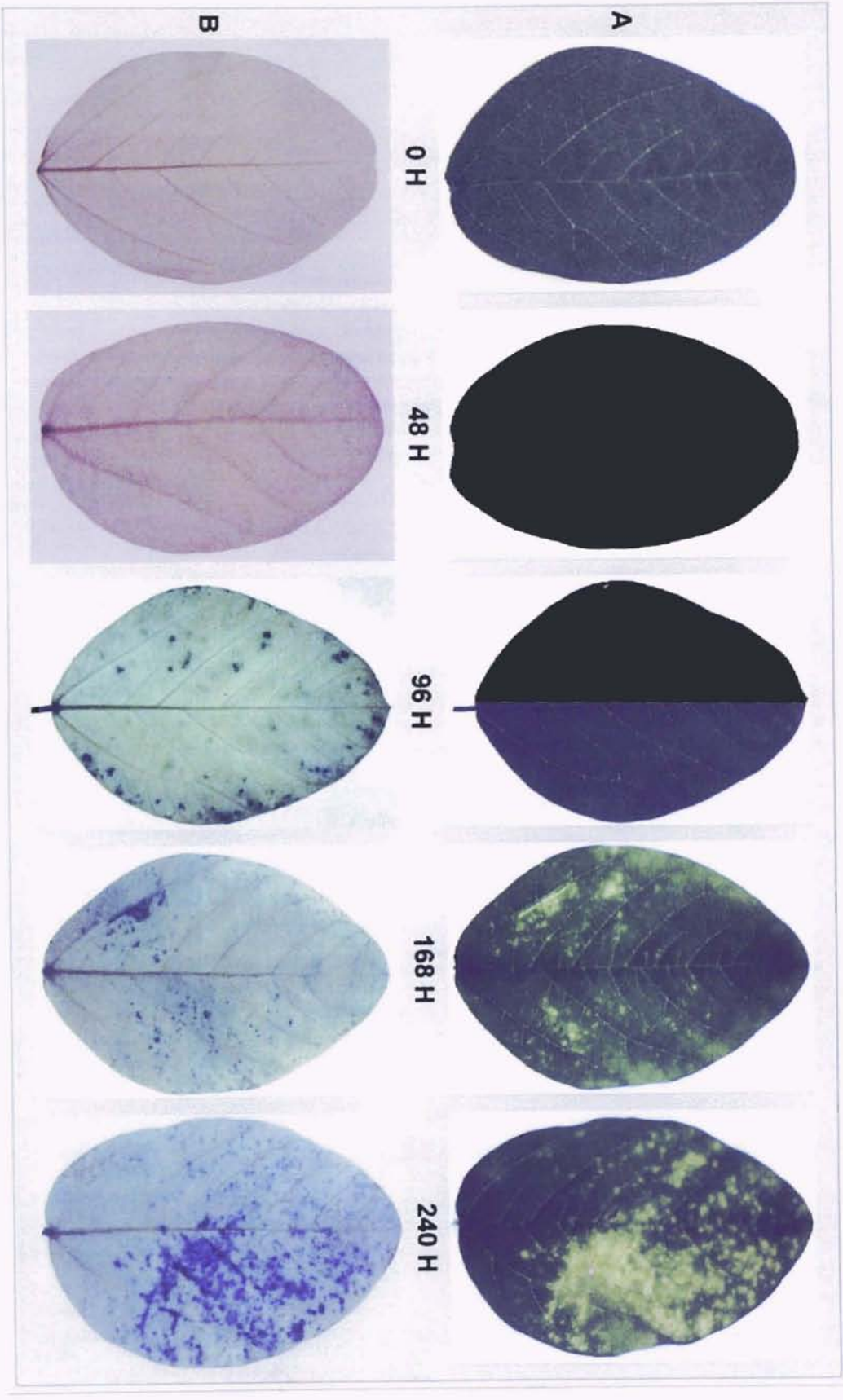
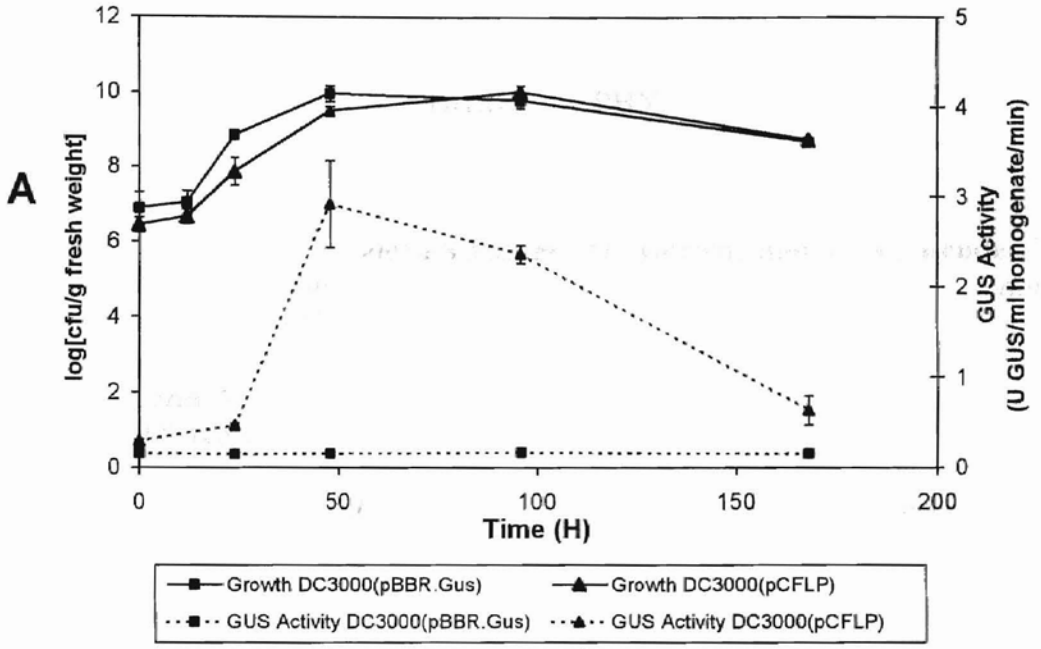
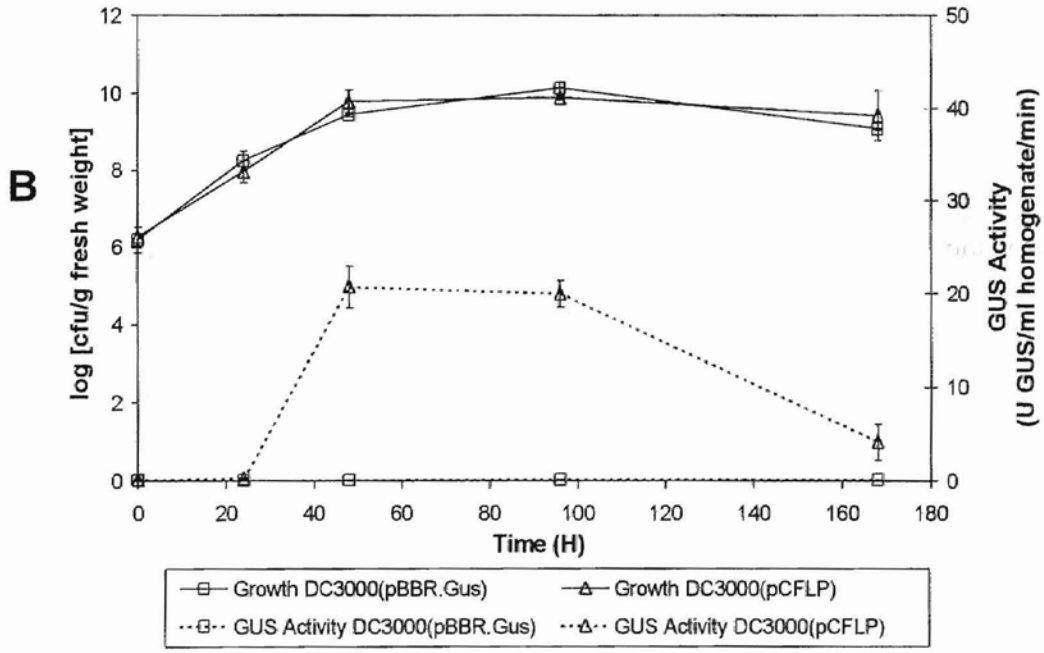

A
B
0 H
48 H
96 H
168 H
240 H

Fig. 9 Bacterial growth *in planta* and fluorometric analysis of GUS activity in tomato **(A)** and in collard **(B)**. Tomato and collard plants were inoculated with DC3000(pBBR.Gus) and DC3000(pCFLP) by spraying and incubated in the growth chamber at 24°C.


A
log[cfu/g fresh weight]
GUS Activity (U GUS/ml homogenate/min)
Time (H)
Growth DC3000(pBBR.Gus)
Growth DC3000(pCFLP)
GUS Activity DC3000(pBBR.Gus)
GUS Activity DC3000(pCFLP)


B
log [cfu/g fresh weight]
GUS Activity (U GUS/ml homogenate/min)
Time (H)
Growth DC3000(pBBR.Gus)
Growth DC3000(pCFLP)
GUS Activity DC3000(pBBR.Gus)
GUS Activity DC3000(pCFLP)

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APPENDIX

DOUBLING TIMES OF DC3000(pBBR.GUS) AND PG4180.N9(pBBR.GUS) AT 21°C

Introduction

Cell growth is the culmination of the orderly interplay of all physiological activities of the cell. Usually the phenomenon of growth is considered from the viewpoint of population increase. Assuming that the time required for a single cell to divide is the same for all cells in a given population, we use the term "doubling time" to refer to the doubling time for the total population. As we know, there are three states in a typical bacterial growth curve, lag, exponential, and stationary phase (Moat and Foster, 1988). In exponential phase, where cell division commences immediately and proceeds in an unhampered fashion for a protracted period of time, the number of cells in the population can be expressed as:

$$b = a \times 2^{n},$$

where b is the number of cells present at a given time, a is the number of organisms present the original inoculum, and n is the number of divisions.

Then,

$$\log_{10} b = \log_{10} a + n \times \log_{10} 2$$

$$\log_{10} b = \log_{10} a + 0.3010\, n$$

Solving the equation for n, the number of generations that occurred between the time of inoculation and the time of sampling is:

$$n = \frac{\log_{10} b - \log_{10} a}{0.3010}$$

The doubling time (t_d) may be determined by dividing the time elapsed (t) by the number of generations (n):

$t_d = t/n$ (Moat and Foster, 1988)

The purpose of this study was to compare the doubling times for *Pseudomonas syringae* pv. *tomato* DC3000 and pv. *glycinea* PG4180.

Materials and Methods

10 ml of HSC broth medium was inoculated with either DC3000(pBBR.Gus) or PG4180.N9(pBBR.Gus), adjusted to O.D.600 = 0.05, and incubated at 21°C, at 280 rpm. At different time points after inoculation, 0.1 ml was sampled, diluted and plated on MG containing chloramphenicol (25 μg/ml) for bacterial counts. The doubling time of each strain was determined according to the trend line of the log phase portion of its growth curve (Fig. 10A, B).

Results and Discussion

Growth curves of DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus) at 21°C

To obtain accurate analysis of the strains used in this project, DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus), not DC3000 and PG4180.N9, were chosen for the study because they contain the vector used for GUS expression studies, and the vector may affect bacterial growth. The growth of DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus) at 21°C was similar (Fig. 10A). Both strains attained log phase within 12 h after inoculation and reached the stationary phase at 24 h (Fig. 10A), suggesting that the differential regulation of COR production in DC3000 and PG4180 is not caused by differential growth.

Doubling times of DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus) at 21°C

Three time points from the log phase in the growth curve of each strain were selected to obtain a linear trend line for calculating the doubling time (Fig. 10B). According to the formula mentioned above,

$$t_d = \frac{0.301\ t}{\log_{10} b - \log_{10} a}$$

and the equations of the trend lines,

$$y = kx + d,$$

where k is the slope of the trend line and d is a constant,

since

$$t = \delta x,$$

$$\log_{10} b - \log_{10} a = \delta y$$

$$\delta y / \delta x = k$$

then

$$t_d = \frac{0.301\ t}{\log_{10} b - \log_{10} a} = \frac{0.301\ \delta x}{\delta y} = \frac{0.301}{k}$$

k was equal to 0.1051 for DC3000(pBBR.Gus) and 0.095 for PG4180.N9(pBBR.Gus) (Fig. 10B). The doubling times *in vitro* of these strains at 21°C were:

t_d [DC3000(pBBR.Gus)] = 0.301/0.1051 = 2.9 h

t_d [PG4180.N9(pBBR.Gus)] = 0.301/0.095 = 3.2 h

The difference in doubling time indicated that DC3000(pBBR.Gus) grew slightly faster than PG4180.N9(pBBR.Gus).

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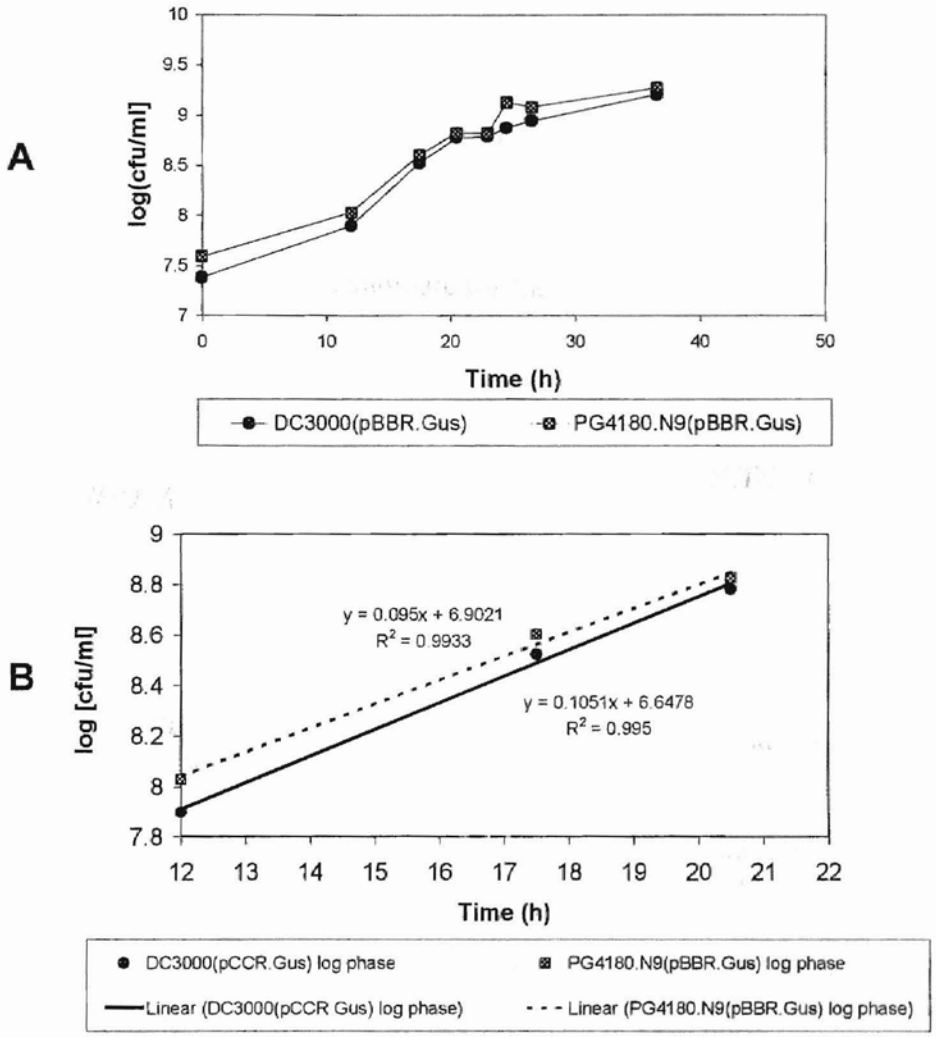

Fig. 10 Doubling times for DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus) at 21°C. **(A)** Growth curves of DC3000(pBBR.Gus) and PG4180.N9(pBBR.Gus) at 21°C. **(B)** The linear trend lines obtained from the selected time points (12, 17.5, and 20.5 h after inoculation) during log phase. The trend line equations and the R-squared values are indicated in the graph.

VITA

Xinwei Wang

Candidate for the Degree of

Master of Science

Thesis: REGULATION OF THE POLYKETIDE PHYTOTOXIN CORONATINE IN *PSEUDOMONAS SYRINGAE*

Major Field: Microbiology, Cell, and Molecular Biology

Biographical:

Personal data: Born in Tianjin, People's Republic of China, On May 23, 1975, the daughter of Wenda Wang and Zhongfeng Wu.

Education: Graduated from the High School No. 1 of Hua Bei Oil Field, Renqu, Hebei, China in July 1993; received Bachelor of Science degree in Biotechnology in the College of Life Sciences from Peking University, Beijing, China in July 1997. Completed the requirements for the Master of Science degree with a major in Microbiology, Cell, and Molecular Biology at Oklahoma State University in May 2001.

Experience: Raised in an oil field in Hebei, China; employed by Oklahoma State University, Department of Microbiology and Molecular Genetics as a graduate teaching assistant and a research assistant, 1997 to present.

Professional Memberships: American Phytopathological Society