ROLE OF CORONATINE IN THE VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000 ON EDIBLE BRASSICAS

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CHAPTER I

INTRODUCTION

Pseudomonas syringae (*P. syringae*) produces a variety of symptoms such as leaf spots, cankers and blights on different plants and is divided into pathovars (pv.) based on host range. The infection of plants by *P. syringae* is a complex process that involves epiphytic colonization, entry into the host plant through natural openings and wounds, establishment in the intercellular space (apoplast), and multiplication and production of disease symptoms. In many *P. syringae* pathovars, low-molecular weight, non host-specific phytotoxins are produced, which induce chlorotic and necrotic symptoms on various host plants (Bender and Scholz-Schroeder, 2004).

Coronatine (COR) is a chlorosis-inducing, non-host-specific phytotoxin produced by multiple *P. syringae* pathovars. The COR molecule has a unique structure and consists of two separate moieties: coronafacic acid (CFA), a polyketide; and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977). The CFA and CMA moieties are linked through an amide bond. COR induces severe chlorosis, hypertrophy, anthocyanin production, ethylene production, alkaloid accumulation, the synthesis of proteinase inhibitors and inhibits root elongation on different plant species (Feys *et al.*, 1994; Lauchli and Boland, 2003; Palmer and Bender, 1995).

In several pathovars of *P. syringae*, COR can contribute to multiplication and lesion formation in various host plants including ryegrass, soybeans, tomato and *Arabidopsis thaliana* (*A. thaliana*).

P. syringae pv. *tomato* DC3000 (*Pst* DC3000) is a model strain for investigating plant-microbe interactions due to its genetic tractability and its pathogenicity on *A. thaliana*. It is also a pathogen of several cultivated plant species, including tomato and crucifers. COR production by *Pst* DC3000 was previously shown to be stimulated in the presence of the host, and the toxin may facilitate the intercellular establishment of the pathogen (Mittal and Davis, 1995; Wang *et al.*, 2002). Genetic studies using the COR-defective mutants of *Pst* DC3000 that lack the ability to produce CFA and/or CMA showed that the intact COR molecule must be produced for maximal virulence on *A. thaliana* (Brooks *et al.*, 2004). However, it is important not to extrapolate the results obtained using *A. thaliana* to make broad generalizations on the reaction of other host plants to *Pst* DC3000 and COR^- mutants, even within the mustard family, Brassicaceae.

Brassicas belong to a large plant family of major economic importance and are used as vegetables, oilseeds, and condiments. In Oklahoma, leafy crucifers like collard, kale, mustard and turnip are important minor crops, and their quality and yield have been negatively impacted by several bacterial diseases (Anonymous, 1994). Since *Brassica* spp. are related to *A. thaliana*, the genomic information available from *A. thaliana* can be utilized to compare the loci that correspond to similar genes in edible brassicas. This approach is powerful

since results obtained in *A. thaliana* can be used to test gene expression in *Brassica* spp.

COR and other virulence factors produced by *P. syringae* can alter major plant defense signaling pathways such as those involving salicylic acid (SA) and jasmonic acid (JA). COR functions in part by promoting bacterial colonization of plant tissue, possibly because it functions as a molecular mimic of methyl jasmonate and suppresses plant defense pathways (Feys *et al.*, 1994; Lauchli and Boland, 2003; Uppalapati *et al.*, 2005; Zhao *et al.*, 2003). Other studies suggest that COR might act as a virulence factor of *P. syringae* by suppressing or delaying the activation of SA-dependent host defense responses (Brooks *et al.*, *in press;* Kloek *et al.*, 2001; Zhao *et al.*, 2003). However, the role of COR in pathogenesis differs with host-pathogen interactions, and the essential mechanisms are still unknown. Since the toxin induces chlorosis, there is potential degradation of chlorophyll and hence rapid induction of the chlorophyllase (Chlase) enzyme in plant tissue.

In the current investigation, we explore the role of COR in the interactions between *Pst* DC3000 and two edible *Brassica* spp., collard and turnip, by utilizing biochemically defined mutants defective in CFA and/or CMA. Symptom development, bacterial growth, pigment changes and defense gene expression were investigated. Furthermore, the responses of both *Brassica* spp. to exogenous application of purified CFA, CMA and COR were also explored.

CHAPTER II

REVIEW OF LITERATURE

(i) *PSEUDOMONAS SYRINGAE*

Biology and symptoms: *Pseudomonas syringae* (*P. syringae*) is a gram-negative plant pathogenic bacterium that produces a wide variety of symptoms in plants such as leaf spots, blights, cankers and wilting (Alfano and Collmer, 1996). The pathogen is subdivided into pathovars or pathogenic variants (pv.), based on host range. Although *P. syringae* interacts with a wide range of host plants, individual strains have a high level of host specificity.

The infection by *P. syringae* is a multifaceted process that initiates with surface or epiphytic colonization. The epiphytic populations on the leaf surface serve as the inoculum source for the disease, and surface establishment directs further disease development and infection on the host plant. Epiphytic growth is ecologically important for the survival and the spread of the pathogen in the field. Successful epiphytes exhibit a range of attributes including chemotaxis, microcolony development, nutrient acquisition, resistance to external stress, and antibiosis. For most plant-associated bacteria, there is a quantitative link between the size of the surface population and the degree of plant disease (Hirano and Upper, 2000). Epiphytic fitness of the bacterium is generally bestowed by traits such as the presence of pili, production of extracellular polysaccharides, and

pigment production. Alginate, which is a co-polymer of *O*-acetylated β-1, 4-linked D-mannuronic acid and L-guluronic acid, has been reported to enhance the epiphytic fitness of *P. syringae* (Keith *et al.*, 2003; Penaloza-Vazquez *et al.*, 2004).

The epiphytic phase is followed by the entry of *P. syringae* into the host plant through natural openings and wounds. The bacteria then establish in the intercellular space (apoplast), where they multiply. Infected leaves show water-soaked lesions that develop into necrotic lesions, which are often surrounded by chlorotic halos (Hirano and Upper, 2000) High leaf humidity and cool temperatures (13 to 25^0C) favor disease development (Preston, 2000).

Type III secretion system: The type III secretion system (TTSS), a major factor in the pathogenicity and epiphytic colonization of susceptible hosts infected by *P. syringae* is encoded by the *hrp*/*hrc* regulon. The TTSS of *P. syringae* encodes the Hrp pilus, which facilitates the delivery of effector proteins and extracellular accessory proteins into the plant apoplast. *P. syringae* accessory or helper proteins are termed as type III chaperones (TTCs) (Guo *et al.*, 2005). Proteins secreted via the TTSS require host cell contact and the presence of chaperones, but not a signal peptide (Galán and Collmer, 1999). The secretion and translocation of effector proteins presumably occur through the *hrp* pilus, which elongates at the distal end by the addition of *hrpA* pilin subunits (Jin *et al.*, 2001; Li *et al.*, 2002). The TTSSs in plant pathogenic bacteria are closely related to the flagellar export systems of bacterial pathogens of animals. Thus, it has been hypothesized that the TTSS might have evolved as an adaptation of the

flagellar apparatus as a conduit for proteins other than flagellin. This would allow the close association of plant pathogens and host cells.

The TTSS of *P. syringae* is encoded by the *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) genes, which are not rigidly conserved among plant pathogenic bacteria. The genes that encode the core components of the TTSS are highly conserved and are known as the *hrc* (*h*ypersensitive *r*esponse and *c*onserved) genes (Bogdanove *et al.*, 1996; Preston, 2000). The importance of the TTSS in the leaf surface establishment of *P. syringae* pv. *syringae* B728a has been established (Hirano *et al.*, 1999). Two *hrp* mutants, *hrpC* and *hrpJ* exhibited very poor epiphytic fitness and caused less disease severity than the wild-type.

Chang *et al.* (2005) conducted a high-throughput screen for type III effector genes of *P. syringae* pvs. *tomato* and *phaseolicola* and defined 29 type III proteins from *P. syringae* pv. *tomato*, and 19 from pv. *phaseolicola* race 6. The study provided full functional annotation of the *hrpL*-dependent type III effector suites from both pathovars and also showed that the high variability of these protein sets apparently reflects the evolutionary selection by various host plants (Chang *et al.*, 2005). The *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) effector database contains 60 ORFs**,** making the bacterium a model for studying plant-pathogen interactions (http://pseudomonas-syringae.org/pst_func_gen.htm).

Virulence in *P. syringae* is multifactorial: The pathogenicity of *P. syringae* depends on diverse factors including**:**

(a) The TTSS and associated effector proteins.

(b) Production of exopolysaccharides (EPS), which contribute to epiphytic fitness, formation of water-soaked lesions, and protection from environmental stress (Keith *et al.*, 2003; Penaloza-Vazquez *et al.*, 2004).

(c) Global regulatory genes, such as *gacA* and *gacS* (also known as *lemA*) (Chatterjee *et al.*, 2003; Preston, 2000).

(d) Low-molecular weight, non-host-specific phytotoxins, which induce chlorotic and necrotic symptoms on a wide variety of host plants (Bender and Scholz-Schroeder, 2004). These non-host-specific toxins are capable of inducing disease symptoms on plants that cannot be infected by the toxin-producing pathogen. These toxins increase the virulence of *P. syringae* and their production amplifies the severity of disease.

Phytotoxins produced by *P. syringae:* Phaseolotoxin is produced by *P. syringae* pvs. *phaseolicola* and *actinidiae*, pathogens that induce halo blight on legumes and kiwi fruit, respectively. The toxin inhibits ornithine carbamoyl transferase leading to the accumulation of ornithine and deficit of intercellular arginine, which ultimately leads to chlorosis. Tox^- mutants of *P. syringae* pv. *phaseolicola* failed to move systemically in bean plants and were less virulent (Peet *et al.*, 1986).

Tabtoxin is a monocyclic β-lactam produced by *P. syringae* pvs. *tabaci, coronafaciens* and *garcae.* The toxin is a dipeptide and consists of tabtoxinine-β-lactam (TβL) linked to threonine by a peptide bond. The hydrolysis of the peptide

bond releases TβL, which irreversibly inhibits glutamine synthetase. The resulting ammonia accumulation in plants disrupts the thylakoid membrane of the chloroplast, uncouples photophosphorylation and eventually leads to chlorosis.

Syringomycins and syringopeptins produced by *P. syringae* pv. *syringae* are necrosis-inducing lipopeptide phytotoxins that contribute to virulence in bacteria–plant interactions. Both toxins have structural and functional similarities and share common biosynthetic, regulatory and secretory mechanisms (Bender and Scholz-Schroeder, 2004; Zhang *et al.*, 1997). Due to the amphipathic nature of syringomycin and syringopeptin, they generate a membrane flux of H^+, K^+ and Ca^{2+}, disrupting the plant membrane potential. This leads to electrolyte leakage, plant cell death and necrosis. Both the toxins are reported to have antimicrobial activity.

P. syringae pvs. *tomato, glycinea, maculicola, atropurpurea* and *morsprunorum* produce coronatine, a chlorosis-inducing non host-specific phyototoxin (Bender *et al.*, 1987; Ullrich *et al.*, 1993; Zhao *et al.*, 2000). This toxin is discussed in greater detail below.

(ii) CORONATINE (COR)

Structure & components: COR has an unusual structure and consists of two distinct moieties: (1) coronafacic acid (CFA), a polyketide; and (2) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977; Mitchell, 1991; Parry *et al.*, 1994a). CFA is synthesized by the polyketide pathway from one unit of pyruvate, one unit of butyrate and three

acetate residues (Parry *et al.*, 1994a). Studies have revealed that the pyruvate is converted to α-ketoglutarate, which is then used as a starter for CFA synthesis (Parry *et al.*, 1996). Parry and co-workers (Parry *et al.*, 1991; Parry *et al.*, 1994b) demonstrated that during CMA biosynthesis, isoleucine is first converted to alloisoleucine, which is then cyclized to form CMA. During the final step in the pathway, CFA and CMA are linked by amide bond to form COR (Bender *et al.*, 1993; Parry *et al.*, 1994a) (Fig. 1). The enzymes involved in this reaction do not have rigid specificity for the amino acid substrate and hence, other coronafacoyl amide toxins such as coronafacoylisoleucine, coronafacoylalloisoleucine, and coronafacoylvaline, are synthesized. However, COR is the most toxic coronafacoyl compound made by COR-producing organisms (Mitchell, 1985a, 1985b; Mitchell and Young, 1985; Mitchell and Ford, 1998).

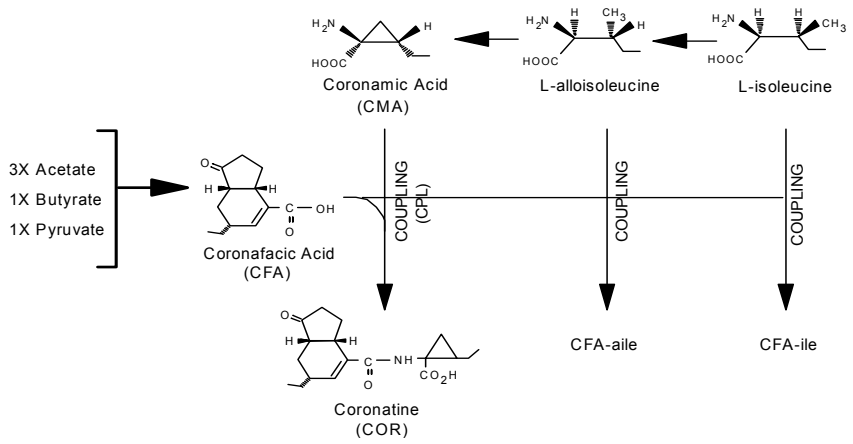


Fig.1. Biochemical pathways involved in the synthesis of coronatine (COR) and coronafacoyl compounds. COR is formed by the coupling (CPL) of a polyketide component, coronafacic acid (CFA), to an amino acid component, coronamic acid (CMA) via an amide bond. CFA is a polyketide derived from three units of acetate, and one unit each of pyruvate and butyrate. CMA is derived from isoleucine via alloisoleucine. CFA can also be coupled to L-alloisoleucine (aile) and L-isoleucine (ile) to form the coronafacoyl analogues, CFA-aile and CFA-ile, respectively.

Biological effects of COR: Diffuse, intense chlorosis is induced on leaf tissue by external application of COR or due to infection by COR-producing *P. syringae* strains. The severe spread of chlorosis in tomato and tobacco plants has been ascribed to the loss of chlorophyll *a* and *b* (Kenyon and Turner, 1992; Palmer and Bender, 1995). Ultrastructural studies have revealed that the chloroplasts of COR-treated tomato have intact membranes; however, the chloroplasts were smaller and stained intensely. The association of COR with the chloroplasts of tomato leaves was proven by the use of COR-specific antiserum in immunolocalization studies (Zhao *et al.*, 2001). Recent studies showed that COR could induce the expression of chlorophyllase, an enzyme in the chlorophyll degradation pathway (Benedetti and Arruda, 2002). The rapid breakdown of chlorophyll could be associated with the rapid senescence of leaves exposed to COR (Tsuchiya *et al.*, 1999).

COR also induces hypertrophy, anthocyanin production, alkaloid accumulation, accumulation of proteinase inhibitors, stimulates ethylene production, and inhibits root elongation on diverse plant species (Feys *et al.*, 1994; Lauchli and Boland, 2003; Palmer and Bender, 1995). In grasses and a few monocots, COR has been associated with apoptosis (cell death) and flavonoid accumulation (Bender and Scholz-Schroeder, 2004).

Involvement of COR in virulence and pathogenicity: COR acts as a virulence factor in some pathovars of *P. syringae* and contributes to bacterial multiplication and lesion formation or development in numerous host plants, including ryegrass, soybeans, tomato and *A. thaliana* (Bender *et al.*, 1987; Budde

and Ullrich, 2000; Mittal and Davis, 1995; Sato *et al.*, 1983). Genetic studies revealed that COR defective (COR^-) strains of *P. syringae* pvs. *tomato, maculicola,* and *atropurpurea* were impaired in their ability to multiply and produced smaller necrotic lesions than the COR-producing strains (Bender *et al.*, 1987; Budde and Ullrich, 2000; Mittal and Davis, 1995; Sato *et al.*, 1983). It is hypothesized that COR acts a molecular mimic of methyl jasmonate and suppresses plant defense pathways, hence promoting the colonization of host tissue (Kloek *et al.*, 2001; Zhao *et al.*, 2003). Nomura *et al.* (2005) portray different models to describe the suppression of basal defense, gene-for-gene resistance and non-host resistance in plants by COR and other virulence factors. This defense suppression is described as the essential step in pathogenesis, and it is likely that COR plays an import role in this process. Other theories proposed for the role of COR in virulence include the promotion of lesion formation and expansion (Bender *et al.*, 1999) as well as the triggering of programmed cell death (Yao *et al.*, 2002). A recent study by Brooks and co-workers (2004) implies that COR has a vital role in the establishment of a successful infection probably by assisting the pathogen in both colonization and persistence in host tissue. Nevertheless, the significance and role of COR in pathogenesis varies with host-pathogen interactions and the mechanisms underlying these activities are not well comprehended.

The structure and function of COR and associated compounds were examined in biological assays, ultrastructural studies and cDNA microarray analysis (Uppalapati *et al.*, 2005). The results showed that conjugation of CFA to

an amino acid is important for chlorosis, changes in chloroplast structure, cell wall thickening, accumulation of proteinase inhibitors, stimulation of anthocyanins, and root growth inhibition. The intact COR molecule has a broader range of activity than its components and also impacts signaling in tomato via the jasmonic acid, ethylene, and auxin pathways.

COR production by *Pst* DC3000: *Pst* DC3000 is a pathogen of several plant species, including tomato, crucifers (cabbage, cauliflower) and the model plant *Arabidopsis thaliana* (*A. thaliana*). The genetic tractability of *Pst* DC3000, its pathogenicity on *A. thaliana*, and the completion of its genomic sequence (Buell *et al.*, 2003) have greatly facilitated the use of *Pst* DC3000 as a model strain for investigating plant-microbe interactions (Preston, 2000; Whalen *et al.*, 1991).

COR production by *Pst* DC3000 is stimulated in the presence of the host. The infection of *A. thaliana* by *Pst* DC3000 induced specific genes in the pathogen, including those involved in COR biosynthesis (Boch *et al.*, 2002). These results agree with an earlier study where transcriptional fusions between *cor* genes and the ice nucleation reporter (*inaZ*) were used to identify plant factors that stimulated *cor* gene expression (Li *et al.*, 1998). Malic, citric, shikimic, and quinic acids present in leaf extracts and apoplastic fluids of tomato were identified as compounds that activate the toxin genes and stimulate COR production (Li *et al.*, 1998). Of all the hosts (tomato, cabbage, soybean and pepper) examined, cabbage was found to have the highest COR-inducing activity. Both qualitative and quantitative differences in plant signal molecules

might contribute to plant-related differences in *cor* gene expression. The production of 25 to 40-fold less COR by *Pst* DC3000 than *P. syringae* pv. *glycinea* PG4180 *in vitro* could be due to the lack of the 'plant factor', which is required for stimulating *cor* gene expression in *Pst* DC3000 (Penaloza-Vazquez *et al.*, 2000).

COR is required for the symptom development or the establishment of *Pst* DC3000 when the pathogen is either dip- or spray-inoculated to *A. thaliana* and tomato. However, when *A. thaliana* or tomato plants are infiltrated with *Pst* DC3000, the toxin is not absolutely required for a successful infection (Mittal and Davis, 1995). These results suggest that COR may have a vital role in facilitating the establishment of the *Pst* DC3000 in the apoplast of susceptible hosts. In another study, Wang *et al*. (2002) compared *cor* gene expression and COR biosynthesis in *Pst* DC3000 and PG4180. Their study suggested that COR was crucial for the establishment of a successful infection by *Pst* DC3000 in collard, whereas COR might not be absolutely required for symptom development and infection of soybean by PG4180.

To appraise the relative contributions of CFA, CMA, and COR in the virulence of *Pst* DC3000 on *A. thaliana,* three isogenic mutants were compared. These mutants were created with Tn*5* insertions in the genes encoding COR biosynthesis (Brooks *et al.*, 2004). The mutant DB4G3 is defective in CFA production and contains a mutation in *cfa6* (encodes a multifunctional polyketide synthase); mutant AK7E2 is defective in CMA production and contains a mutation in *cmaA* (encodes a gene that catalyzes the formation of adenylated

alloisoleucine, a precursor to CMA). Mutant DB29 is a double mutant, defective in the synthesis of both CFA and CMA, and contains mutations in both *cfa6* and *cmaA*. The virulence phenotypes of these mutants were compared with wild-type *Pst* DC3000 on *A. thaliana*. Both the single and double mutants were equally impaired in their ability to elicit symptoms and multiply in *A. thaliana*, leading the authors to conclude that the intact COR molecule must be produced for maximal virulence on *A. thaliana*. However, it is important to note that other host plants may respond differently to *Pst* DC3000 and COR^- mutants (discussed below).

(iii) THE MODE OF ACTION OF COR: CLUES FROM PHYTOHORMONES

Jasmonic acid pathway: Jasmonates are fatty acid derivatives that play a pivotal role in many biological activities in plants. In *A. thaliana*, jasmonates inhibit root elongation, while they also promote senescence, pollen development and anther dehiscence (Devoto and Turner, 2003; Weber, 2002). Jasmonic acid (JA) and methyl jasmonate (MeJA) are plant defense molecules produced in response to attack of certain herbivores and certain pathogens. *A. thaliana* defective in the biosynthesis or perception of JA failed to show defense responses after insect or pathogen attack (Creelman and Mulpuri, 2002). The JA pathway is turned on in response to the attack by fungal pathogens or wounding or herbivory. Linolenic acid is released from the damaged plant plasma membrane, which in turn is converted to 12-oxo-phytodioenic acid (12-OPDA) by the sequential action of lipoxygenase (LOX), allene oxide synthase, and allene oxide cyclase. Lipoxygenase (*LOX* gene) is a common marker used to follow the expression of JA signaling. OPDA is converted to JA by a reductase and three

steps of β-oxidation. OPDA, JA, and other octadecanoid molecules can act upon various molecular targets to activate gene expression and defense responses (Lauchli and Boland, 2003) (Fig. 2). Jasmonates have both stimulatory and inhibitory effects on plant morphology and physiology (Creelman and Mulpuri, 2002). When plants are wounded, rapid JA biosynthesis and massive transcriptional reprogramming occurs, leading to the expression of many wound-related and pathogenesis-related proteins (Liechti and Farmer, 2002).

New cyclopentenones have been found in plants, and they display varied signaling functions. Two mechanisms of gene expression by jasmonates have been illustrated recently: (a) activation and suppression of various overlapping sets of genes by JA and OPDA; (b) alteration of gene expression via the electrophilic activities of the cyclopentenone ring in JA-related molecules. Current studies have identified *A. thaliana* MAP kinase 4 as a positive regulator of JA-induced gene expression (Farmer *et al.*, 2003).

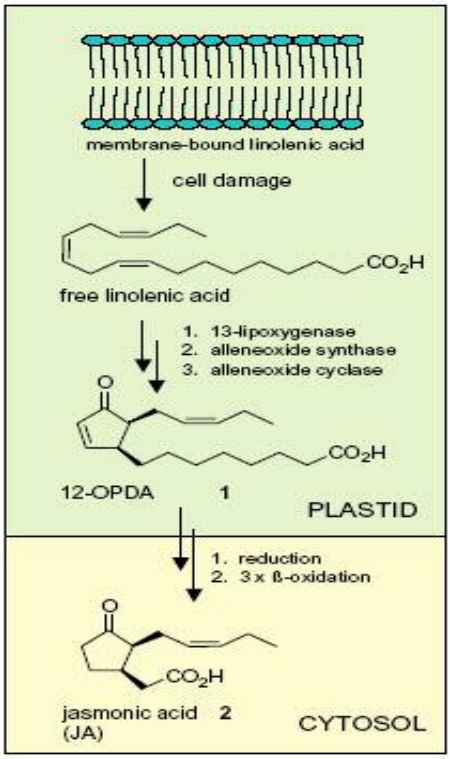


Fig.2. Jasmonic acid biosynthetic pathway (Lauchli and Boland, 2003).

COR is thought to be an analog of octadecanoid signaling molecules that modulate defense reactions in plants (Weiler *et al.*, 1994). It was noted that COR functions as a structural and functional analog of JA, MeJA and their C_{18} precursor, OPDA (Feys *et al.*, 1994; Lauchli and Boland, 2003). COR and MeJA were found to have similar, but not identical functions in tomato where they modulate multiple phytohormone pathways(Uppalapati *et al.*, 2005). An *A. thaliana* mutant insensitive to COR (*coi1*) was also insensitive to MeJA (Benedetti *et al.*, 1998; Feys *et al.*, 1994). The *COI1* allele was shown to encode a 66-kDa protein containing an N-terminal F-box motif and a leucine-rich repeat domain (Weber, 2002; Xie *et al.*, 1998). F-box proteins in eukaryotes function as receptors that employ regulatory proteins as substrates for ubiquitin-mediated degradation. COI1 presumably functions in protein degradation by means of the E3 ubiquitin ligase complex, which could eventually modulate the abundance of proteins that control the expression of JA/COR-responsive genes (Devoto *et al.*, 2002; Xu, 2002). There seems to be a common site of action for COR and octadecanoids, and they may bind to common receptors or interacting proteins. How COR might function as a virulence factor by mimicking MeJA in plant-bacteria interactions is still unknown. The identification of additional genes induced or suppressed by COR might yield information on novel genes that are also regulated by octadecanoids and will further augment our understanding of the role of the octadecanoids in plant defense.

Salicylic acid pathway: Salicylic acid (SA) plays a critical signaling role in the activation of local and systemic plant defense responses after pathogen

attack. There are two proposed pathways for SA biosynthesis in plants; one pathway originates from phenylalanine and a second from shikimate (Fig. 3). Phenylalanine ammonia lyase, the core enzyme in phenylpropanoid metabolism converts phenylalanine to trans-cinnamic acid, which is in turn converted to benzoic acid. In the second pathway, SA is synthesized from shikimate though chorismate and isochorismate (Shah, 2003).

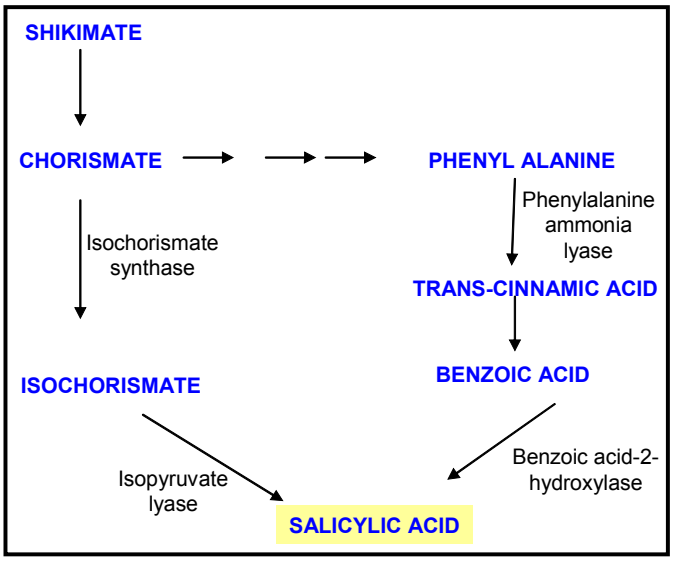


Fig.3. Salicylic acid biosynthetic pathway (modified from Shah, 2003).

Increased levels of endogenous SA, as well as the exogenous application of SA, activated pathogenesis-related (PR) proteins and enhanced resistance to a broad range of pathogens (Ryals *et al.*, 1996). Application of the commercially available SA analog, benzo (1, 2, 3) thiadiazole-7-carbothioc acid S-methyl ester (BTH), potentiated defense gene expression, accumulation of hydrogen peroxide and subsequent cell death, processes that are associated with elevated plant defense (Fitzgerald *et al.*, 2004).

Genetic studies have shown that SA is required for the rapid activation of defense responses and for the establishment of systemic acquired resistance (SAR) (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). SAR is characterized by an increase in endogenous SA, transcriptional activation of the *PR* genes and enhanced resistance to a wide spectrum of virulent pathogens. Increasing SA concentrations in plants either by endogenous synthesis or exogenous application of SA induced SAR(Metraux *et al.*, 1990). Synthetic SA analogs such as BTH and 2, 6-dichloroisonicotinic acid (INA) are also effective inducers of SAR (Görlach *et al.*, 1996).

The significance of SA in plant defense is further authenticated by the use of transgenic plant lines that degrade SA as well as by the isolation of plant mutants that are defective in SA production. For example, many investigators have utilized transgenic plants that are unable to accumulate SA due to the expression of the SA-degrading enzyme, salicylate hydroxylase, which is encoded by the *nahG* gene (Delaney *et al.*, 1994). The importance of SA in plant defense is further supported by the use of plant mutants such as the *A. thaliana npr1-1* (*n*on-expressor of *p*athogenesis *r*elated genes) line, which is impaired in the production of SA-related transcripts. For example, the *npr1-1*mutant of *A. thaliana* was extremely susceptible to infection with *P. syringae*, thus supporting a role for PR proteins and SA in modulating defense in this pathosystem (Cao *et al.*, 1994). Another mutant that has proven helpful for demonstrating the importance of SA in defense is the *A. thaliana coi1* (*co*ronatine-*i*nsensitive) mutant. Kloek *et al*. (2001) studied the interaction of the *coi1* mutant with *Pst*

DC3000 and showed a rapid and strong expression of the *PR-1* gene, which is a well-accepted marker for following SA accumulation. The authors concluded that the resistance in *coi1* plants could be due to enhanced signaling through the SA-dependent defense pathway. This study also provides a clue that COR might act as a virulence factor of *P. syringae* by restraining or delaying the activation of SA-dependent host defense responses.

Several additional studies provide further evidence that COR functions by suppressing the plant defense response. In tomato, Zhao *et al.* (2003) observed the repression of *PR* gene expression in tomato plants inoculated with the COR-producing *Pst* DC3000 but not with a COR^- mutant. In another study, SA levels were quantified and shown to be lower in tomato inoculated with *Pst* DC3000 in comparison to a COR^- mutant (Uppalapati *et al.*, in preparation). In a recent study, SA defective *A. thaliana* plants were used in combination with wild-type *Pst* DC3000 and COR^- strains to show that COR was required to overcome or suppress SA-dependent defenses and for normal disease symptom development in *A. thaliana* (Brooks *et al*., *in press*). New studies analyzing the molecular and biochemical action of effectors associated with the TTSS show that these proteins contribute to bacterial pathogenicity by hampering plant defense signal transduction.

Ethylene is another signaling molecule that plays an important role in disease resistance depending upon the pathogen and plant species. It has been proposed that ethylene causes leaf abscission and thus restricts the spread of the pathogen (Bleecker and Kende, 2000.). Ethylene signaling is modulated

through a pathway that includes a MAP kinase and a transcriptional cascade leading to biological responses. Ethylene-insensitive (*ein*) or ethylene-resistant (*etr*) mutants of *A. thaliana* with minor or no response to ethylene treatment are used to study the ethylene signal transduction pathway and its role in plant disease resistance. Previous reports have shown the production of ethylene in COR-treated tissue (Kenyon and Turner, 1992). One possible clue to this activity is the structural similarity of CMA and aminocyclopropyl carboxylic acid (ACC), which is an intermediate in the ethylene pathway (Bender *et al.*, 1999). (Fig.4). However, recent investigations using seedling assays, ultrastructural studies and gene expression profiling have shown that CMA does not function as an analog of ACC in tomato (Uppalapati *et al.*, 2005).

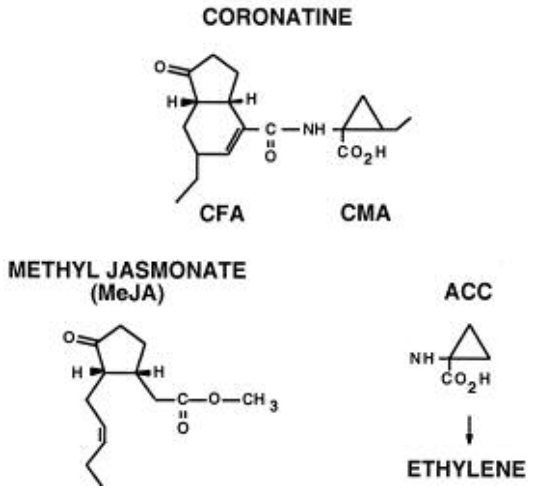


Fig.4. Structures of coronatine (COR), methyl jasmonate (MeJA) and aminocyclopropyl carboxylic acid (ACC) (Brooks *et al.*, 2004).

Cross-talk between pathways: Further clues regarding the mechanism of action for COR can be extrapolated from the interaction of different signaling pathways. It is important to note that the JA, SA and ethylene signaling pathways do not function independently. Rather, they are engaged in an intricate signaling complex in which the different pathways manipulate each other through positive and negative regulatory interactions. Furthermore, the roles of these hormones vary with the particular host-pathogen interaction (Hoffman *et al.*, 1999).

The interactions between the SA and JA pathways are complex, and there is evidence for both positive and negative interactions between these two signaling pathways. Studies with tobacco and *A. thaliana* have suggested that the major form of interaction between these pathways is mutual antagonism (Norman-Setterblad *et al.*, 2000); in other words, when activity in one pathway is elevated, the other pathway is negatively impacted. The inhibitory effect of SA on JA signaling is well-demonstrated (Doares *et al.*, 1995; Doherty *et al.*, 1988; Gupta *et al.*, 2000; Peña-Cortés *et al.*, 1993); however, several reports also indicate that JA antagonizes the SA pathway (Kachroo *et al.*, 2001; Kloek *et al.*, 2001; Niki *et al.*, 1998).

Several proteins have now been identified that modulate signaling between the SA and JA pathways, including WRKY70, NPR1, and NPR4. The WRKY70 transcription factor presumably functions as an activator of SA-induced genes and a repressor of JA-responsive genes, thus integrating signals from these mutually antagonistic pathways (Li *et al.*, 2004). The WRKY family of transcription factors is characterized by a DNA binding domain that contains the

highly conserved amino acid sequence WRKYGQK. Transcription factors in the WRKY family have been shown to confer disease resistance and trigger the expression of defense-related genes during SAR (Eulgem *et al.*, 2000; Robatzek and Somssich, 2002). NPR1, a key regulator of the SA pathway and PR protein expression, has been shown to modulate the cross-talk between SA- and JA-dependent defense pathways (Pieterse and Van Loon, 2004). Liu *et al.* observed that NPR4 protein (which shares 36% identity with NPR1) positively regulates the expression of SA and JA responsive genes and hence may modulate cross-talk between these two pathways (Liu *et al.*, 2005). In addition, microarray analysis of *A. thaliana* has revealed more than 50 defense-related genes coordinately regulated and induced by SA and JA (Schenk *et al.*, 2000). *P. syringae* may use COR and TTSS effectors to activate the jasmonic acid (JA) pathway and thereby inhibit or delay salicylic acid–mediated plant responses (Fig. 5) (Mudgett, 2005).

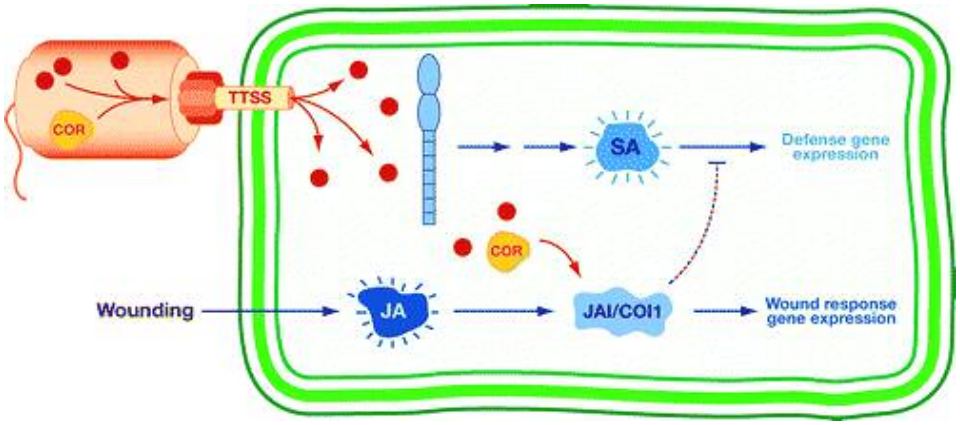


Fig.5. Model for *P. syringae*- host plant interaction (Mudgett, 2005)

It is also important to note that the ethylene pathway communicates with both the SA and JA pathways. For example, transcriptional activity in the JA and

ethylene pathways is often coordinately regulated (Glazebrook *et al.*, 2003; Kunkel and Brooks, 2002; Lorenzo *et al.*, 2003; Schenk *et al.*, 2000) . Ethylene is known to interact with the SA pathway in a synergistic manner, which has been revealed by microarray experiments and *nahG* transgenic of *A. thaliana* (O'Donnell *et al.*, 2003; Schenk *et al.*, 2000).

In summary, there is ample evidence that plant defense responses may be fine-tuned by separate signaling pathways so that plants install the proper combination of defenses against specific pathogens and control their temporal expression.

Chlorophyllase (Chlase): Chlorophyllase is presumably the first enzyme in the chlorophyll degradation pathway (Matile *et al.*, 1999). Pathogen attack can result in the release of chlorophyll (from thylakoid membrane), which has to be degraded to avoid the accumulation of reactive oxygen species. Therefore, the degradation of chlorophyll is vital in order to avoid cellular damage.

Chlase is rapidly induced in response to wounding and treatment with MeJA or COR; consequently it is thought to be associated with JA dependent defenses (Benedetti *et al*., 1998; Brooks *et al*., *in press*). Two Chlase-encoding genes were identified and characterized from *A. thaliana* (Benedetti and Arruda, 2002; Tsuchiya *et al.*, 1999). *A. thaliana* plants silenced by RNAi for *AtCLH1*, which encodes Chlase in *A. thaliana*, are resistant to *Erwinia carotovora*. The *AtCLH1*-silenced plants showed increased levels of SA as well as enhanced induction of SA-dependent defense genes like *PR-1* and *PR-2*. This suggests the potential involvement of Chlase in promoting JA-dependent defense and

repressing SA-mediated plant defense in *A. thaliana* (Kariola *et al.*, 2005). These authors also propose a hypothetical model for the role of Chlase in plant defense, where reactive oxygen species and the SA and JA-mediated defense pathways each participate in determining the outcome of the response to different pathogens.

(iv) BRASSICAS/CRUCIFERS

Importance of edible brassicas. The Cruciferae/Brassicaceae or 'mustard family' is a large plant family of major economic importance that contains an assorted variety of crop plants. On an economic basis, brassicas are grouped into oilseed, vegetable, and condiment crops. *Brassica* spp. are a valuable source of dietary fiber, vitamin C, and contain other health-promoting factors such as anticancer compounds (Fahey and Talalay, 1995). Leafy crucifers like collard, kale, mustard and turnip are important minor crops in Oklahoma. About 600 ha of these vegetables are grown every year during both spring and fall cropping seasons and are harvested up to three times per cropping season (Anonymous, 1994). Among the brassicas, turnip *(Brassica rapa* L., cultivar 'All Top') and collard (*B. oleracea* L., cultivar 'Vates') are among the main varieties grown in Oklahoma with yields recorded up to 6 to 12 tons/acre. These crucifers have been damaged by many diseases leading to a reduction in product quality and economic loss. Although some fungal diseases cause problems in *Brassica* spp., bacterial pathogens have been the primary disease-causing agents of leafy greens in Oklahoma.

Bacterial diseases of brassicas/crucifers. Several bacterial pathogens cause diseases on crucifers or brassicas (Lelliott and Stead, 1987). These include *Xanthomonas campestris* pv. *campestris* (black rot of crucifers) (Pammel, 1893; Williams, 1980), *X. campestris* pv. *raphani* (bacterial leaf spot of radish and turnip) (White, 1930), *P. syringae* pv. *maculicola* (pepper spot of crucifers and bacterial leaf spot of brassicas) (McCulloch, 1911), *P. viridiflava* (leaf spots of various crucifers), *P. cichorii, P*. *marginalis,* and *X. campestris* pv. *aberrans*. A new, unidentified pathovar of *P. syringae* was reported to cause leaf spot and blight of broccoli raab (*Brassica rapa* subsp. *rapa)* (Koike *et al.*, 1998) The pathogen was closely related to *P. syringae* pvs. *coronafaciens* and *maculicola*. Further characterization of the pathogen was done by host range analyses, phage sensitivity, ice nucleation and BOX-PCR, and the pathogen was identified as *P. syringae* pv. *alisalensis* pv. nov., causal agent of leaf spot and blight of broccoli raab (Cintas *et al.*, 2002). The first report of bacterial leaf spot caused by *P*. *syringae* pv. *maculicola* on leafy crucifers in Oklahoma was published by (Zhao *et al.*, 2000) and includes the identification and characterization of fluorescent *Pseudomonas* spp. isolated from leafy crucifers in Oklahoma.

***P. syringae* pv. *maculicola*:** *P. syringae* pv. *maculicola* causes bacterial leaf spot (pepper leaf spot) of crucifers (McCulloch, 1911). The disease is widespread throughout the world and has been reported in countries where cauliflower and other crucifers are grown. The distinctive symptoms induced by the pathogen are small brown spots with irregular edges surrounded by chlorotic halos. The spots enlarge and coalesce creating large necrotic areas. *P. syringae*

pv. *maculicola* strains are classified into three groups based on different types of lesions on cauliflower and tomato leaves (Wiebe and Campbell, 1993). These groups consist of those that induce chlorotic (CL), water-soaked (WS), and necrotic lesions (NL). The WS lesion is a small, brown, water-soaked lesion without a chlorotic halo; the CL is similar to WS, but with a chlorotic halo. The NL is a small, dark grey, dry and sunken spot. Bacterial leaf spot is more prevalent during high rainfall and in coastal valleys and causes major economic losses in cauliflower and broccoli (Campbell *et al.*, 1987). *P. syringae* pv. *maculicola* causes disease on cabbage, cauliflower, turnip, broccoli, kohlrabi, mustard, Chinese cabbage, rape, brussel sprouts, and radish and also infects tomato (Cuppels and Ainsworth, 1995).

P. syringae pv. *maculicola* and *P. syringae* pv. *tomato* (causal agent of bacterial speck of tomato) are distinct, but closely related pathovars. It has been proposed that these two pathovars be combined based on pathological and physiological tests (Takikawa *et al.*, 1992). *Pst* DC3000 and *P. syringae* pv. *maculicola* have similarities in phenotype, DNA homology, and COR production (Zhao *et al.*, 2000), and the taxonomic status of these two pathogens remains debatable (Cuppels and Ainsworth, 1995; Wiebe and Campbell, 1993).

Zhao and co-workers examined the response of different edible *Brassica* spp. to *P. syringae pv. maculicola* and *pv. tomato*. Several *P. syringae* strains examined showed a differential response on collard and turnip, and a preliminary study suggested that the two *Brassica* spp. respond differently to COR (Zhao *et al.*, 2000; Zhao *et al.*, 2002).

Brassica – *A. thaliana* relatedness: Because of its relatedness to the model plant Arabidopsis, the genus *Brassica* is an exceptional system for studying genome divergence and relatedness. *A. thaliana* is not an economically important crop; however, its small physical size, rapid life cycle, small genome, abundant seed production, easy manipulation, genetic tractability and the availability of numerous mutants and genetically transformed lines make it a 'model organism' in plant research. Genetic studies of *B. oleracea*, *B. napus* and *B. rapa* have also been performed and several molecular maps within the genera have been published (Bohuon *et al.*, 1998; Lan and Paterson, 2000). The genomic information available from model species such as *A. thaliana* can be compared to the non-model species (e.g. brassicas), and the loci that correspond to the candidate genes mapped can be easily recognized. Sequence identity between the *B. oleracea* and *A. thaliana* genomes is much higher in the coding regions (85%) than in non-coding regions, and so *A. thaliana* cDNA microarrays could be used to explore global gene expression in brassicas (www.tigr.org/tdb/e2k1/bog1/ and http://brassica.bbsrc.ac.uk/). Thus, comparative genomics could advance research in the more complex *Brassica* genomes by exploiting the *A. thaliana* chromosome sequence maps (Arabidopsis Genome Initiative, 2000).

(v) GOALS OF THE PRESENT STUDY

In this study, I investigated the role and mode of action of COR in the virulence of *Pst* DC3000 on edible species of brassicas. The main objectives of the study were:

(1) Determine whether COR functions as a virulence factor for *Pst* DC3000 in collard and turnip.

(2) Examine the influence of COR on modulating selected defense pathways in brassicas.

(3) Investigate how collard and turnip respond to exogenous application of COR and its components, coronafacic acid and coronamic acid.

CHAPTER III

The Phytotoxin Coronatine from *Pseudomonas syringae* pv. *tomato* DC3000 Functions as a Virulence Factor and Influences Defense Pathways in Edible Brassicas

SUMMARY

The phytotoxin coronatine (COR), produced by *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, is proposed to be a virulence factor that facilitates the successful infection of host plants by suppressing host defense mechanisms. In the present investigation, we analyzed the role of COR and its components, coronafacic acid (CFA) and coronamic acid (CMA) in the virulence of *Pst* DC3000 on collard and turnip, two important edible brassicas. These host plants were inoculated with well-defined mutants of *Pst* DC3000 that were defective in either CFA, CMA or both components of the COR molecule and monitored for symptom development, bacterial colonization, chlorophyll and anthocyanin content. Furthermore, we investigated the influence of COR on major plant defense signaling pathways. Our results suggest that COR contributes to the symptom development in both hosts and multiplication of *Pst* DC3000 in turnip. The CFA component was found to contribute to the development of symptoms in turnip. Gene expression analysis conducted using real-time quantitative PCR indicated that *Pst* DC3000 suppresses the salicylic acid pathway in collard, while

COR producing *Pst* DC3000 and the mutant AK7E2 (CFA^+ CMA^-) function at least partially to suppress the SA pathway in turnip. *Pst* DC3000 promoted the expression of the JA response gene, *LOX2* in both collard and turnip. However, in turnip, *Pst* DC3000 and AK7E2 induced expression of *LOX2*. *CORI1*, which encodes chlorophyllase, was expressed in collard leaves inoculated with the wild-type COR^+ strain and not with the biosynthetic mutants. Turnip leaves inoculated with either *Pst* DC3000 or AK7E2 showed an increase in *CORI1* gene expression. Our results suggest that COR may modulate plant defense in both brassicas, but the CFA moiety functions differentially with respect to defense in the two hosts. The defense responses were slightly delayed in turnip when compared to those in collard. With respect to symptom development, the two brassicas differed substantially. Unlike in collard, the symptoms were delayed in turnip and no water soaking or anthocyanin production were observed. The late symptom development, pigment changes and the difference in bacterial population kinetics indicate that COR has totally different effects in these two host plants with respect to disease development. This is the first study to document differences between *Pst* DC3000 and COR-defective mutants in economically important brassicas using well-defined COR biosynthetic mutants.

INTRODUCTION

Plants have developed an array of defense mechanisms in response to attack by microbial pathogens, including the activation of hormone signaling pathways, rapid oxidative burst, and the accumulation of phytoalexins. Salicylic

acid (SA) is a compound known to activate local and systemic plant defenses. The significance of SA in plant defense is further authenticated by *A. thaliana* mutants that are defective in SA production (*npr1-1* mutants) and transgenic lines of Arabidopsis that do not accumulate SA, such as those expressing the *nahG* gene, which encodes salicylate hydroxylase, an enzyme that degrades SA to catechol (Brooks *et al.*, *in press;* Cao *et al.*, 1994; Delaney *et al.*, 1994). The activation of the SA pathway leads to the expression of pathogenesis-related (PR) proteins; hence *PR* genes are used to monitor SA-mediated defenses (Glazebrook, 2001; Ryals *et al.*, 1996).

Jasmonic acid (JA) and methyl jasmonate (MeJA) are plant defense molecules produced in response to herbivore attack, wounding, and some bacterial and fungal pathogens. When plants are wounded, JA is synthesized rapidly and extensive transcriptional reprogramming occurs, resulting in the expression of many defense-related proteins (Liechti and Farmer, 2002). Lipoxygenase (LOX) is commonly used as a marker to follow the activation of JA pathway. The JA pathway has been associated with the virulence of *Pst* DC3000 during its interactions with *A. thaliana* and tomato. One explanation for this phenomenon is that *Pst* DC3000-produced COR may function of as an analog of JA and/or suppress the SA pathway (Block *et al.*, 2005; Heck *et al.*, 2003; Zhao *et al.*, 2003).

Successful pathogens have evolved mechanisms to enter host tissue, suppress defense mechanisms, multiply and induce disease symptoms. *Pst* DC3000, a model strain for studying the molecular basis of plant-pathogen

interactions, causes leaf spots on *A. thaliana*, tomato and *Brassica* spp. (Cuppels, 1986; Preston, 2000; Zhao *et al.*, 2000). Coronatine (COR) is a chlorosis-inducing, non-host-specific phytotoxin produced by several *P. syringae* pathovars, including *Pst* DC3000 (Bender *et al.*, 1999; Bender and Scholz-Schroeder, 2004). The COR molecule has an unusual structure and consists of two components, coronafacic acid (CFA), a polyketide; and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977; Parry *et al.*, 1994). The major biological effects of COR include hypertrophy, chlorosis, accumulation of proteinase inhibitors, increased anthocyanin levels, stimulation of ethylene production and inhibition of root growth (Feys *et al.*, 1994; Lauchli and Boland, 2003; Palmer and Bender, 1995). Chlorosis being the major effect, chlorophyll degradation can be monitored using Chlase (CORI1) gene expression in plants.

COR is involved in the virulence of some strains of *P. syringae*. Studies in soybean, tomato and *A. thaliana* have shown that the toxin acts as a virulence factor and contributes to chlorosis and lesion expansion (Bender *et al.*, 1999; Brooks *et al.*, 2004; Penaloza-Vazquez *et al.*, 2000). It has been demonstrated that COR interferes with SA-mediated defenses in *A. thaliana* (Kloek *et al.*, 2001). The CFA moiety of COR has structural and functional similarity to JA, MeJA (Fig. 5; Chapter II), and their C_{18} precursor, 12-oxo-phytodienoic acid (OPDA) (Brooks *et al.*, *in press;* Feys *et al.*, 1994; Lauchli and Boland, 2003; Uppalapati *et al.*, 2005). In *A. thaliana*, COR stimulates jasmonate responses, which presumably suppress SA-dependent defenses, and it has been proposed

that COR functions via both SA-dependent and SA-independent mechanisms (Block *et al.*, 2005; Brooks *et al.*, *in press;* Nickstadt *et al.*, 2004; Uppalapati *et al.*, 2005).

It is important not to make broad generalizations regarding the mode of action for COR based on findings obtained with *A. thaliana*. Thus it is critical to analyze the role of COR and how it manipulates defense signaling in other plant species infected by *P. syringae*. In this study, biochemically-defined COR-defective mutants were used to explore the function of COR and its components in the virulence of *Pst* DC3000 on commercially important, edible brassicas, e.g. collard and turnip. Many COR-producing strains of *P. syringae* were identified from *Brassica* spp. in Oklahoma and their survival and inoculum sources were also determined (Zhao *et al.*, 2000; Zhao *et al.*, 2002). In a previous study, Wang *et al*. (2002) investigated the transcriptional activity of a *cor* biosynthetic gene and showed that it was expressed very early in collard leaves, suggesting a potential role for COR in the infection of collard plants.

In the present study, the relatedness of *A. thaliana* to edible brassicas at the genomic level was exploited to analyze gene expression in collard and turnip in response to *Pst* DC3000 and a series of well-defined COR biosynthetic mutants. Differences between collard and turnip in response to COR were thoroughly analyzed by inoculating these hosts with *Pst* DC3000 and three COR-defective mutants and monitoring bacterial growth, symptom development, chlorophyll degradation, anthocyanin accumulation and gene expression in response to the different bacterial strains. The results show that COR is a

virulence factor in both brassicas, but contributes to the multiplication of *Pst* DC3000 only in turnip. Real-time quantitative PCR analysis indicated that the SA pathway was suppressed in turnip and collard inoculated with the COR-producing wild-type *Pst* DC3000. However, the results obtained from symptom analysis, population kinetics and defense gene expression suggest some striking differences between turnip and collard in response to *Pst* DC3000.

Experimental Procedures

Plant material

Seeds of collard (*Brassica oleraceae* var. *viridis* L. cv. Vates) and turnip (*B. rapa* var. *utilis* (DC) Metzg. cv. Alltop) were obtained from Twilley Seed Company (Hodges, SC). The plants were grown in Metro-Mix® 200 (Sierra Horticultural Products Co., Marysville, OH) and maintained in a growth room at 24-25^{0}C, 35-40% relative humidity (RH) with a photoperiod of 12 h. Bacterial inoculation experiments were conducted using four-week old plants.

Bacterial strains and inoculation

Pst DC3000 and mutants AK7E2 (CFA$^+$ CMA$^-$; *cmaA*::Tn*5*), DB4G3 (CFA$^-$ CMA$^+$; *cfa6*::Tn*5*), and DB29 (CFA$^-$ CMA$^-$; *cfa6*-*cmaA* double mutant) (Brooks *et al.*, 2004) were used in this study. *Pst* DC3000 and the mutants were grown at 28^{o}C for 24 h on mannitol glutamate (MG) agar supplemented with the following antibiotics (µg/mL): rifampicin, 100; kanamaycin, 25; and spectinomycin, 100. Bacterial cells were suspended to an OD_{600}=0.1 (~10^7 cfu ml^{-1}) in sterile distilled

water, and the surfactant Silwet L77 (Osi Specialties Inc., Danbury, CT) was added to the bacterial inoculum at a concentration of 0.025%. Four-week-old plants were spray-inoculated with an airbrush (55.2 kPa) until leaf surfaces were evenly wet. After inoculation, the plants were incubated in a growth chamber with a photoperiod of 12 h at 25^{o}C. The relative humidity (RH) was 90% for the first 48 h after inoculation and 70% for the remainder of the experiment.

Symptoms and bacterial growth

The plants were monitored for disease symptoms and bacterial colonization during a seven day period. Visible symptoms such as chlorosis, water soaking, necrosis, and anthocyanin accumulation on plants inoculated with *Pst* DC3000 were compared to plants inoculated with the COR^{-} mutants. Disease severity was scored according to the percentage of leaf area showing symptoms using a 0-4 rating scale as follows: 0, no disease; 1, 1 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, 76 to 100%. The number of leaves showing individual symptoms out of the total number of leaves was also calculated for each host/strain interaction to calculate percentage infection.

The total as well as internal population of all bacterial strains was estimated 0, 1, 3, and 6 days after inoculation. Random leaf samples were collected at each sampling time and each leaf was dissected along the midrib. One half of each leaf was used to assess the total bacterial population and the remaining half was surface sterilized with 15% H_2O_2 for 5 min and then rinsed three times in sterile distilled water to estimate the internal population. Leaf discs (4 replications, 10 mm diameter) were removed from individual leaf sections and

macerated in sterile distilled water with a mortar and pestle. Dilutions of leaf homogenate made in sterile distilled water were plated onto MG medium containing the appropriate antibiotics. The plates were then incubated at 25^0C for 48 h, and bacterial colonies were counted. The experiments were repeated at least three times with similar results.

Pigment estimation

The levels of chlorophyll and anthocyanin in the inoculated leaves were estimated 7 days after inoculation. Leaf discs (10 mm diameter) were macerated in 1 ml of ice-cold ethyl acetate/acetone (1:1 v/v) solvent mixture. This was diluted (1:33 ratio) in the same solvent mixture and absorbance was read at 665 and 649 nm to estimate chlorophylls *a* and *b*, respectively.

For anthocyanin determination, the method described by Gould *et al*. (2000) was used. Leaf discs (10 mm diameter) were agitated gently in 1 ml of a solution containing 3M HCl: H_2O: methanol (1:3:16 volume ratio) in the dark for 24 h at 4^oC. The solution was removed, absorbance was read at 530 and 653 nm, and anthocyanin was estimated as A_{530}-0.24 $A_{653.}$ Subtraction of 0.24 A_{653} compensates for the small overlap in absorbance at 530 nm by the chlorophylls (Gould *et al*., 2000). The readings were plotted as the percentage chlorophyll or anthocyanin present in inoculated leaves relative to the mock-treated control (100%). The experiments were repeated at least three times with similar results and the standard deviation was included.

Northern blot analysis

Total RNA was extracted from inoculated leaves collected at different time points (0, 12, 24 and 48 h) using TRIZOL (Sigma) reagent, a mono-phasic solution of phenol and guanidine isothiocyanate. During sample (100 mg of leaf tissue) homogenization, TRIZOL disrupts cells and dissolves cell components, while maintaining the integrity of the RNA. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains entirely in the aqueous phase, which is recovered by precipitation with isopropyl alcohol.

Ten µg of RNA (extracted as described above) was electrophoresed on formaldehyde gels, transferred to nylon membranes, and fixed to membranes using UV light (Stratalinker® UV Crosslinker). Hybridization probes were prepared using the Random Primer Labeling kit (Invitrogen Life Technologies, Carlsbad, CA). *A. thaliana* cDNA clones containing genes indicative of the SA pathway (*PR-1*), the JA pathway (*LOX2*) and chlorophyllase (*CORI1*) were used as probes. The probes were received from the lab of Dr. Barbara N. Kunkel (Washington University, St. Louis, MO). The *LOX2* probe was a 1.0 kb *Eco*RI-*Bam*HI cDNA fragment from *A. thaliana LOX2* (*AtLOX2*) gene and was cloned into pZL1 (Brooks *et al.*, *in press*). The *AtLOX2* gene was cloned and characterized (Genbank Accession No. L23968) by Bell and Mullet (1993). The *PR-1* probe contained a 750 bp *Eco*RI-*Xho*I fragment with the complete coding sequence of the *A. thaliana PR-1* gene (Genbank Accession No. NM_127025.2) (Brooks *et al.*, *in press;* Uknes *et al.*, 1992). The *CORI1* probe contained a 1.15 kb *Eco*RI-*Xho*I fragment with the complete coding sequence of *A. thaliana*

CORI1 gene (Genbank accession number- AF021244) (Benedetti *et al.*, 1998; Brooks *et al.*, 2005 in press). The cDNA (50 ng) was labeled with ^{32}P (50 µCi), hybridized at 45^{o}C overnight, and washed twice (2X SSC at room temperature and 1X SSC at 45^{o}C). RNA blots were exposed for 3 to 4 days and analyzed using a phosphorimager.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from leaves inoculated with *Pst* DC3000 and the COR^{-} mutants at 0, 0.5, 1, 3, 6, 12, 24 and 48 h after inoculation. The quantity of RNA was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by gel electrophoresis. The extracted RNA was treated with 1 µL (1 U per µL) of amplification grade DNAse I (Invitrogen) to avoid contamination by genomic DNA. DNAse I was inactivated by adding 1 µL of 25 mM EDTA and incubating at 65^{o}C. 1 µg of the DNAse-treated RNA was used to synthesize cDNA using the Superscript ^{TM} First Strand Synthesis System (Invitrogen). RNA not incubated with reverse transcriptase was included as a negative control in the unlikely event that samples contained any remaining genomic DNA. The quantity of cDNA was estimated using the NanoDrop ND-1000 spectrophotometer, and the volume was adjusted to 100 ng per reaction.

Primers for *PR-1*, *LOX2*, *CORI1* and 18S rRNA (included as a constitutively expressed control) were designed using D-LUX^{TM} Designer software available from Invitrogen (www.invitrogen.com/lux). The primer sets were predicted to amplify fragments ranging in size from 60 to105 bp (Table 1).

One primer from each set was labeled with the fluorophore JOE (6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein). All the primers were designed from regions of *A. thaliana* genes showing the highest homology with *Brassica* spp. The known sequences of all the genes from *A. thaliana* were aligned with available ESTs of *Brassica* spp. The Basic Local Alignment Search Tool (BLAST) from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find regions of similarity between sequences.

Table 1. Fluorogenic LUX primer pairs used for quantitative real-time PCR.

Target gene	**Labeled LUX primer (forward orientation)**	**Unlabeled primer (reverse orientation)**	**Predicted size (bp)**
PR-1	CACTGACCAAGTTGTTTGGAGAAA GTCAG[JOE]G	CCATTGTTACACCTCACTTTGG	66
LOX2	GAACCTTGGTGGCCTGTCCT	CTACGAGAGGTGACCCATGCAA TCG[JOE]AG	80
CORI1	CGAAGTCAAAGCTCACCTACCAAC TT[JOE]G	TGGCCCACGAGTGAGGTGTA	66
18S rRNA	GACCTGGGAAGTTTGAGGCAATAA CAGG[JOE]C	TGTCGGCCAAGGTGTGAACT	105

PCR was performed using the HotStarTaq Master Mix Kit (250 U Kit; Qiagen Inc., Valencia, CA), which included HotStarTaq DNA polymerase (250 U), $MgCl_2$ (final reaction concentration 1.5 mM), and 200 μM of each dNTP. The total reaction volume for real-time PCR was 25 μl, and each reaction contained 3 μM of the gene specific primers (1 μl), 12.5 μl of the HotStar Taq Master Mix, and 100 ng of the cDNA template (2 μl). The reactions were incubated at 95°C for

15 min, and then cycled (40 X) at 95°C for 15 s, 60°C for 1 min and 72°C for 34 s. Reactions were performed in a 96-well format in a spectrofluorometric thermal cycler (ABI PRISM 7700 Sequence Detector System, Applied Biosystems). The amount of fluorescence as a function of PCR cycle was plotted using the ABI PRISM 7700 SDS software, and the threshold cycle (C_t) values were obtained. The C_t values for all target genes were first normalized using the gene encoding 18S rRNA to get ΔC_t. Gene expression was calculated by the comparative C_t method, in which the relative amount of target gene expression is compared to the gene expression at time zero (calibrator) to get the $\Delta\Delta C_t$. Target gene expression was represented as the relative fold increase in the transcript level with reference to gene expression at time zero. The relative differences in target genes were calculated according to the $\Delta\Delta C_t$ mathematical model (Pfaffl, 2001).

RESULTS

Symptom development on collard and turnip inoculated with *Pst* DC3000 and COR-defective mutants

The role of COR as a virulence factor was investigated by inoculating collard and turnip with *Pst* DC3000 and the three COR^- mutants. On collard plants inoculated with *Pst* DC3000, chlorotic, water-soaked (WS) lesions appeared three days after inoculation. Anthocyanins (apparent as a purple pigment) accumulated on the lower surface of *Pst* DC3000-inoculated leaves.

Between three and six days, the chlorotic lesions enlarged and eventually developed complete chlorosis and necrosis. Severe chlorosis, water-soaking, necrosis and anthocyanin production were apparent on the seventh day after inoculation (Fig. 6). Out of the total number of collard leaves sprayed with *Pst* DC3000, 65, 60 and 53 percent of the number of leaves exhibited chlorosis, WS and necrotic lesions, and anthocyanin accumulation, respectively (Fig. 6A-D and Table 2).

Leaf samples collected from *Pst* DC3000-inoculated leaves exhibited 75-100% chlorosis, necrosis and WS lesions and were rated as a 4 on the disease severity scale (Table 2). The extent of anthocyanin production was rated as 3. The leaves inoculated with the single mutants (AK7E2 and DB4G3) showed mild chlorosis, necrosis, WS lesions and anthocyanin accumulation. Collard leaves sprayed with the double mutant (DB29) showed negligible chlorosis, necrosis and WS lesions and mild anthocyanin levels, and these symptoms were rated as 1 (Table 2).

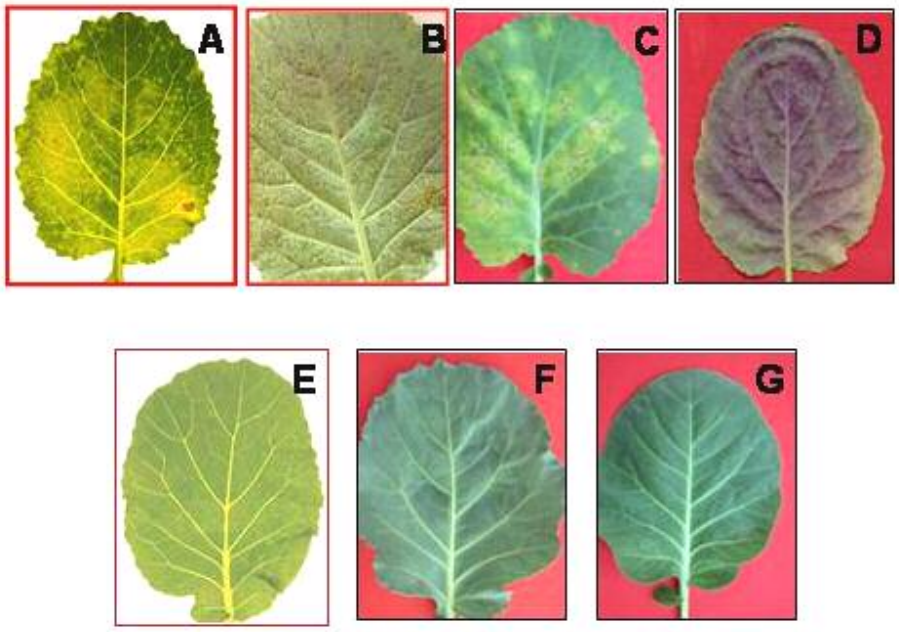


Fig. 6. Symptoms on collard leaves sprayed with *Pst* DC3000 (panels **A-D**), AK7E2 (*cmaA* mutant; panel **E**), DB4G3 (*cfa6* mutant; panel **F**), and DB29 (*cmaA cfa6* mutant, panel **G**). The plants were sprayed with 10^7 CFU/ml of inoculum and incubated in a growth chamber as described in Experimental Procedures. The leaves were photographed 7 days after inoculation.

Table 2. Percentage of disease in collard inoculated with *Pst* DC3000 and COR-defective mutants.

Bacterial strains	**Chlorosis**		**Necrosis & Water soaking**		**Anthocyanin**	
	Disease %	Rating	Disease %	Rating	Disease %	Rating
Pst DC3000	65 ± 5.6	*4*	60 ± 4.5	*4*	53 ± 6.0	*3*
DB4G3 (CFA^-)	3 ± 1.2	*1*	5 ± 1.5	*1*	4 ± 2.2	*1*
AK7E2 (CMA^-)	8 ± 2.3	*1*	9 ± 3	*1*	5± 2.6	*1*
DB29 ($CFA^- CMA^-$)	3 ± 1.5	*1*	4 ± 1	*1*	2 ± 1.6	*1*

In turnip, severe symptoms were induced by *Pst* DC3000, and 57 and 54% leaves showed chlorosis or necrosis, respectively (Fig. 7A, Table 3). Unlike in collard, on which symptoms were apparent beginning three days after inoculation, symptoms were not visible on turnip until the fifth day after

inoculation. Furthermore, turnip leaves inoculated with *Pst* DC3000 did not exhibit WS lesions or anthocyanin production, symptoms that were observed in collard. The CFA$^-$ mutant DB4G3 (*cfa6*) and the CFA$^-$ CMA$^-$ double mutant DB29 (*cfa6-cmaA*) did not produce highly noticeable symptoms on turnip (Fig. 7C, D). However, AK7E2 (*cmaA* mutant) induced severe chlorosis and necrosis in turnip leaves (Fig. 7B, Table 3). These results indicate that collard and turnip differ considerably with respect to symptom development when inoculated with *Pst* DC3000 and the COR$^-$ mutants.

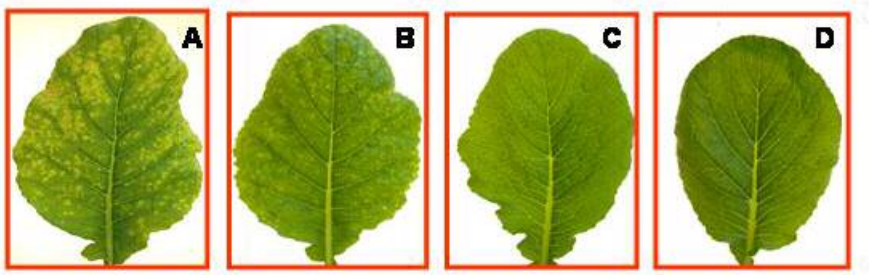


Fig. 7. Symptoms on turnip leaves sprayed with: **A**, *Pst* DC3000; **B,** AK7E2 (*cmaA* mutant); **C**, DB4G3 (*cfa6* mutant) and **D**, DB29 (*cmaA cfa6* mutant). The plants were spray-inoculated with 10^7 CFU/ml of inoculum and incubated in a growth chamber as described in Experimental Procedures. Leaves were photographed 7 days after inoculation.

Table. 3. Percentage of disease in turnip inoculated with *Pst* DC3000 and COR-defective mutants.

Bacterial strains	**Chlorosis**		**Necrosis**		**Anthocyanin**	
	Disease %	Rating	Disease %	Rating	Disease %	Rating
Pst DC3000	57 ± 2.6	*4*	54 ± 5.1	*3*	0	*0*
DB4G3 (CFA$^-$)	4 ± 2.5	*1*	4 ± 1.5	*1*	0	*0*
AK7E2 (CMA$^-$)	35 ± 4	*3*	21 ± 4.5	*2*	0	*0*
DB29 (CFA$^-$ CMA$^-$)	4 ± 2.6	*1*	3 ± 1.2	*1*	0	*0*

Growth of *Pst* DC3000 and COR$^-$ mutants on collard and turnip

The population dynamics of *Pst* DC3000 and the COR-defective mutants were evaluated to determine whether COR contributed to the multiplication of the bacteria *in planta*. Interestingly, the total (Fig. 8) and internal (data not shown) populations of *Pst* DC3000 and the three COR-defective mutants were not significantly different on collard during the six-day sampling period, which suggests that COR does not facilitate the multiplication of *Pst* DC3000 in collard.

Pst DC3000 and AK7E2 (*cmaA* mutant) showed significantly higher populations in turnip at three days after inoculation (10^8 CFU/g leaf tissue) (Fig. 9). *Pst* DC3000 maintained a higher total population on turnip throughout the experiment, while the AK7E2 population declined such that at day 6 it was not distinguishable from those of DB4G3 and DB29. There was no significant difference in either the population of DB4G3 and DB29 (*cfa6* and *cmaA-cfa6* mutants, respectively). Growth of these mutants *in planta* was consistently lower than *Pst* DC3000, and their population was significantly lower than *Pst* DC3000 by the sixth day of sampling. The higher population of *Pst* DC3000 in turnip indicates that COR is important for the persistence of the bacterium in turnip but not collard; thus these two brassicas respond quite differently to the pathogen.

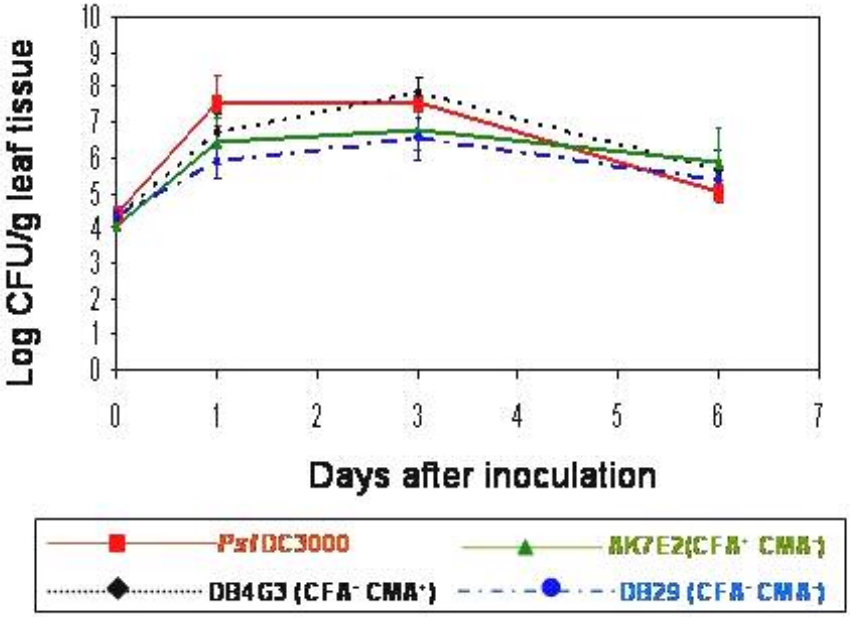


Fig. 8. Total populations of *Pst* DC3000, AK7E2 (*cmaA*), DB4G3 (*cfa6*) and DB29 (*cmaA-cfa6*) on collard leaves. The symptoms on these plants are shown in Fig. 6. Collard plants were inoculated as described in Experimental Procedures. The experiments were repeated at least twice and the vertical bars show the standard deviation.

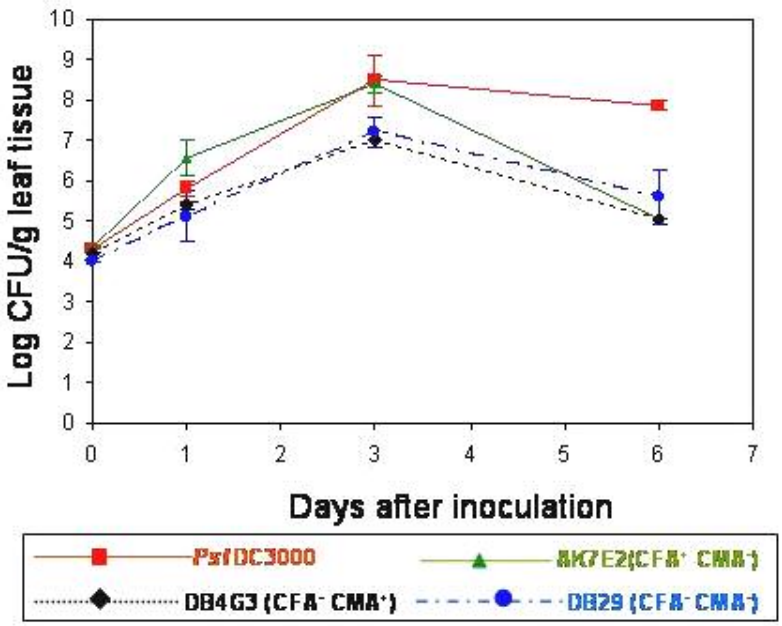


Fig.9. Total populations of *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant) on turnip leaves. The symptoms on these plants are shown in Fig. 7. Turnip plants were inoculated as described in Experimental Procedures. The experiments were repeated at least twice and the vertical bars show the standard deviation.

Chlorophyll and anthocyanin estimation

Inoculation with *Pst* DC3000 induced changes in the pigmentation of leaves, and this was quantified by measuring chlorophyll and anthocyanin content. Chlorophyll levels in *Pst* DC3000-inoculated collard leaves were only 20% of the mock-inoculated control leaves, which was significantly lower (Fig. 10A). This finding is consistent with the observation of chlorosis on collard leaves inoculated with *Pst* DC3000 (Fig. 6A). Collard leaves inoculated with the COR-defective mutants showed only a slight reduction in chlorophyll content compared to those inoculated with *Pst* DC3000 (Fig. 10A), and this relationship was consistent with the little or no chlorotic symptoms in these leaves (Fig. 6E-G).

The level of anthocyanin was approximately threefold higher in collard leaves inoculated with COR-producing *Pst* DC3000 than the collard leaves treated with any of the mutants and the control (Fig. 10B). This is in agreement with the accumulation of purple pigmentation in collard leaves inoculated with *Pst* DC3000 (Fig. 6D). The collard leaves inoculated with the single and double mutants showed very low anthocyanin levels, which were comparable to those in the water-inoculated control (Fig. 10B). These data are consistent with the visual symptoms, as we do not observe anthocyanin production in collard leaves sprayed with the COR-defective mutants (Fig. 6E-G).

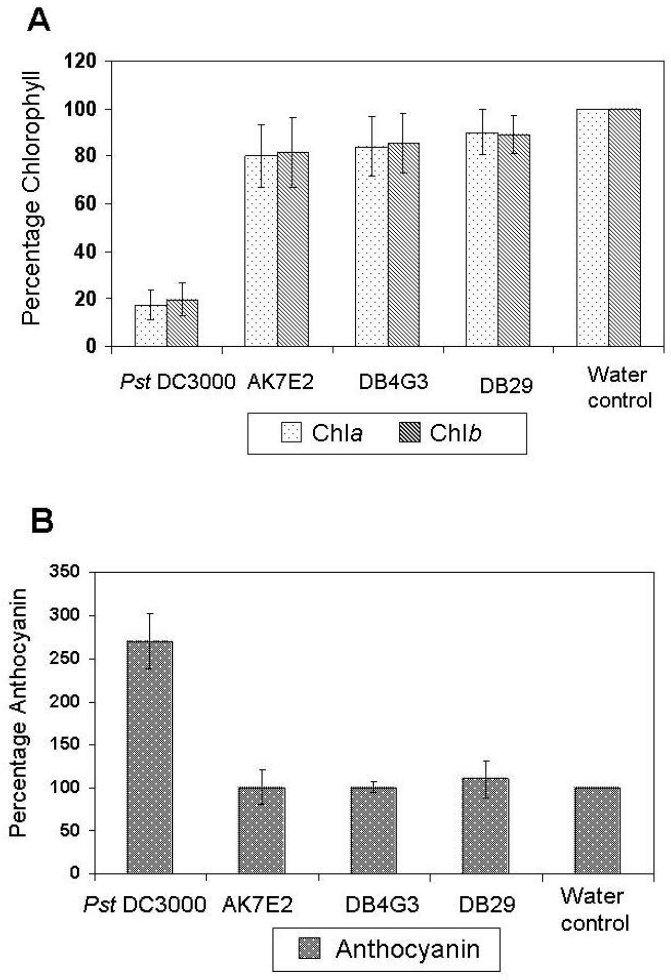


Fig.10. Chlorophyll and anthocyanin levels in collard leaves inoculated with *Pst* DC3000 and the COR-defective mutants. **A,** Chlorophyll content in collard leaves inoculated with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant). **B,** Anthocyanin levels in collard leaves inoculated with *Pst* DC3000, AK7E2, DB4G3 and DB29. Pigments were analyzed 7 days after inoculation, and the analysis was performed three times with similar results.

The chlorophyll content of turnip leaves inoculated with the wild-type DC3000 and the CMA$^-$ mutant was reduced to ~30% and ~70% as compared to the mock-inoculated control, respectively (Fig. 11). These results are consistent with the chlorosis observed on turnip leaves inoculated with these strains (Fig.7A and B). There were no significant differences in the chlorophyll levels of turnip inoculated with DB4G3 and DB29. Anthocyanin levels in turnip inoculated with DC3000 and the COR$^-$ mutants were not significantly different from each other (data not shown). This agrees with the visible anthocyanin pigmentation in turnip plants.

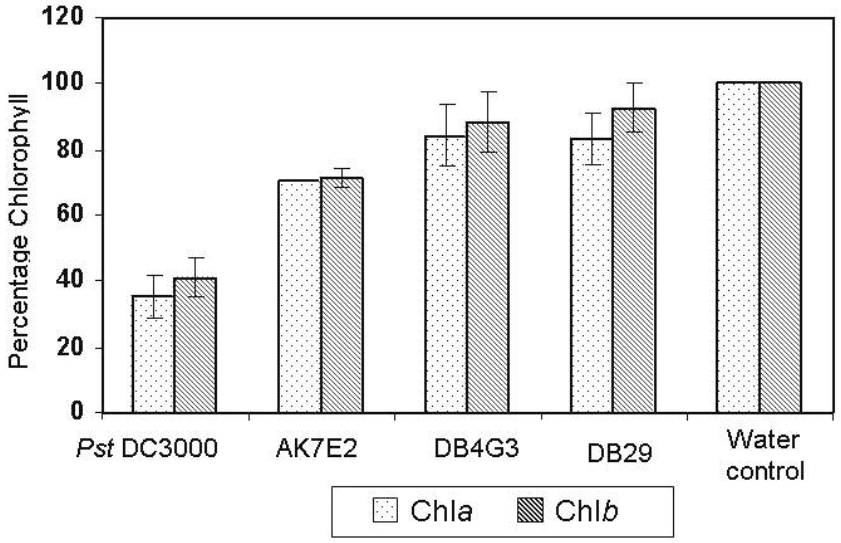


Fig.11. Chlorophyll content in turnip leaves inoculated with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant). Pigments were analyzed 7 days after inoculation, and the analysis was performed three times with similar results.

Gene expression analysis

The expression of genes indicative of the SA pathway (*PR-1*), JA pathway (*LOX2*) and chlorophyllase enzyme (*CORI1*) were monitored in both *Brassica* spp. treated with *Pst* DC3000 and the COR biosynthetic mutants. In both collard and turnip, a weak expression of *CORI1* was detected 48 h after inoculation with

Pst DC3000, but not after inoculation with the COR-defective mutants (Fig. 12A and Fig. 13A, respectively). Even though the signals in northern blots were not extremely clear, the results agree with the observations of chlorosis and low chlorophyll content in collard inoculated with *Pst* DC3000, but not in those inoculated with the COR-defective mutants. Although northern blot analysis was not sensitive enough to detect *CORI1* induction in turnip in response to AK7E2 (*cmaA* mutant), inoculation with this mutant induced chlorosis and lowered the chlorophyll levels in turnip (Figs. 7, 11). It has been hypothesized that COR contributes to the virulence of *P. syringae* by suppressing host defense mechanisms in *A. thaliana* and tomato (Kloek *et al.*, 2001; Zhao *et al.*, 2003). To test the modulation of host defense mechanisms by COR in collard and turnip, we monitored the expression of *LOX2* and *PR-1*, which are indicative of transcriptional activity in the JA and SA pathways, respectively. *LOX2* expression was detected at all the time points analyzed in both collard and turnip plants inoculated with *Pst* DC3000 and the three COR-defective mutants (Fig. 12B; Fig. 13B). Thus, analysis by RNA blots did not show a significant difference in *LOX2* expression in *Pst* DC3000-inoculated plants as compared to the COR-defective mutant-inoculated plants, which would be predicted if the JA pathway was activated in response to COR production.

The transcriptional activity of *PR-1* (indicative of activity in the SA pathway) was observed only at 48 h and only in turnip leaves inoculated with DB29 (*cmaA-cfa6* mutant) (Fig. 13C). This finding suggests that *PR-1* is suppressed in turnip inoculated with *Pst* DC3000, and supports the hypothesis

that COR functions in part by down-regulating the SA pathway. However, since inoculation with AK7E2 (*cmaA* mutant) and DB4G3 (*cfa6* mutant) also failed in *PR-1* expression, we are forced to conclude that both parts of the COR molecule are required for this effect. Expression of *PR-1* was not detected by RNA blot analysis in collard leaves inoculated with *Pst* DC3000 or the COR-defective mutants (data not shown).

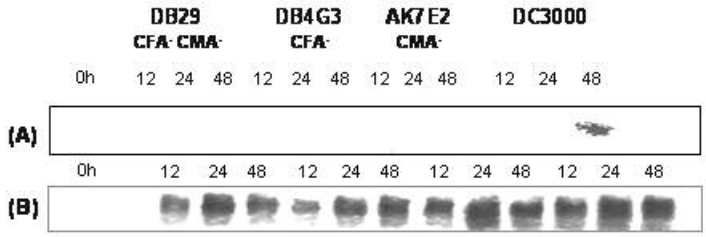


Fig.12. Gene expression in collard by RNA blot analysis. Expression of (**A**) *CORI1* and (**B**) *LOX2* at 0, 12, 24 and 48 h after inoculation with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant).

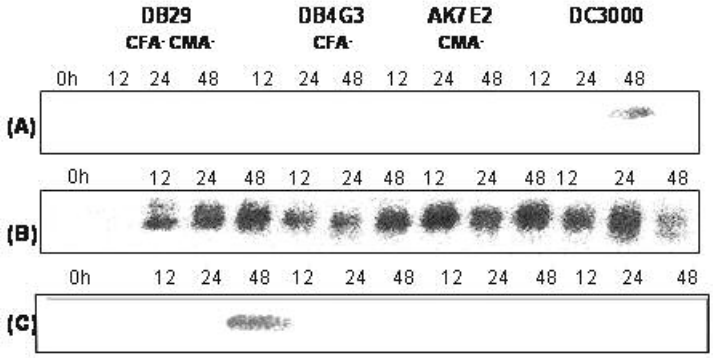


Fig. 13. Gene expression in turnip by RNA blot analysis. Expression of (**A**) *CORI1*, (**B**) *LOX2* and (**C**) *PR-1* genes at 0, 12, 24 and 48 h after inoculation with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant).

The results described above suggest that COR stimulates chlorophyll degradation and may modulate SA-mediated defenses in turnip. Collectively, these results point to activation of the JA pathway in both collard and turnip in response to *Pst* DC3000 and the COR-defective mutants. Surprisingly, differential expression of *LOX2* was not detected by RNA blot analysis; in other words, we predicted that *LOX2* expression would be higher in *Pst* DC3000-inoculated leaves, but this was not observed by RNA blot analysis. Also, COR is thought to down-regulate SA-dependent defense responses. Thus we would predict that expression of *PR-1*, which is indicative of gene expression in the SA pathway, would be repressed in plants inoculated with the COR-producing wild-type strain *Pst* DC3000, but expressed in response to COR-defective mutants. However, *PR-1* expression was not detected by RNA blot analysis in collard plants, regardless of the strain or time point. Nevertheless, in turnip leaves inoculated with the double mutant DB29 (*cmaA-cfa6*), expression of *PR-1* was observed at 48 h (Fig. 13C).

Although the results from RNA blot analysis show expression of the genes of interest to some extent, we could not draw logical conclusions due to either faint signal or lack of signals from the blots as well as the lack of reproducibility of the results. This could be due to different reasons. For example, *LOX2* and *CORI1* are 'early expressed' genes in plants, so the time points analyzed in the RNA blots may not have been those at which the highest transcript abundance occurred. Secondly, the probes from *A. thaliana* contained full-length cDNA, including the 5' and 3' UTR (untranslated region), which are not highly

homologous to the *Brassica* UTR. Finally, the probe sizes (> 750 bp) may have been too large to get a reliable signal in northern blot analysis.

Since the results from northern blot analysis were not very consistent or conclusive, the expression of *PR-1, LOX2* and *CORI1* were monitored in collard and turnip by real-time quantitative PCR (RT-qPCR), which is a much more sensitive technique than RNA blot analysis. The primers were designed from *A. thaliana* coding regions that are highly homologous to those of *Brassica* spp. Since these primers would anneal to a smaller region than that recognized by the probes used in the northern blots, and since primer annealing is followed by amplification, the results should be more reliable. Initially, gene expression was monitored at the same time points used in the RNA blot analysis. Based on some preliminary experiments, very early time points were also included. Thus, gene expression was evaluated at a series of time points (0, 0.5, 1, 3, 6, 12, 24 and 48 h post inoculation) for each gene. Furthermore, in experiments in which *LOX2* expression was monitored, a 15 min time point was included.

When RT-qPCR was used to monitor gene expression in collard, a rapid, transient induction of *PR-1* was observed 6 h after inoculation with DB29 (*cmaA-cfa6* mutant). Interestingly, this surge was not observed with either of the single mutants (AK7E2 or DB4G3), suggesting that both components of the COR molecule are required for suppression of *PR-1* in collard. The expression of *PR-1* was significantly lower (~1000-fold) in collard plants inoculated with *Pst* DC3000, AK7E2, and DB4G3 (Fig. 14A). These data suggest the suppression of the SA pathway by COR and its components, CFA and CMA in collard. It is likely

that SA-mediated defenses are suppressed in collard inoculated with *Pst* DC3000, and the lack of *PR-1* induction in AK7E2 and DB4G3-inoculated plants indicates that both CMA and CFA are required for *PR-1* suppression in collard.

Expression of the JA/wound induced gene, *LOX2*, was observed in collard plants as early as 15 and 30 min after inoculation with *Pst* DC3000. The expression peaked at 30 min and was over 300-fold higher than expression of *LOX2* in collard inoculated with the single and double mutants (Fig. 14B). The RT-qPCR data indicate that COR production by *Pst* DC3000 does stimulate a rapid increase in *LOX2* activity. LOX activity occurs early in the JA pathway and hence this gene is expressed early in collard plants inoculated with the wild-type COR-producing bacterium, but not in plants inoculated with COR-defective mutants.

In collard, maximal expression of the chlorophyllase-encoding gene, *CORI1*, occurred 1 h after inoculation with COR-producing *Pst* DC3000. Induction of *CORI1* was not observed in collard inoculated with the COR-defective mutants. Transcription was over 100-fold greater in plants inoculated with the COR-producing bacterium *Pst* DC3000 than in the COR-defective mutants (Fig. 14C). These results correlate with the chlorosis that developed on *Pst* DC3000-inoculated collard leaves and with the absence of chlorotic lesions in collard leaves inoculated with the mutant strains (Fig. 6). Furthermore, these results agree with the significant decrease in chlorophylls *a* and *b* that was apparent in collard leaves inoculated with *Pst* DC3000, but not the COR-defective mutants (Fig. 10A).

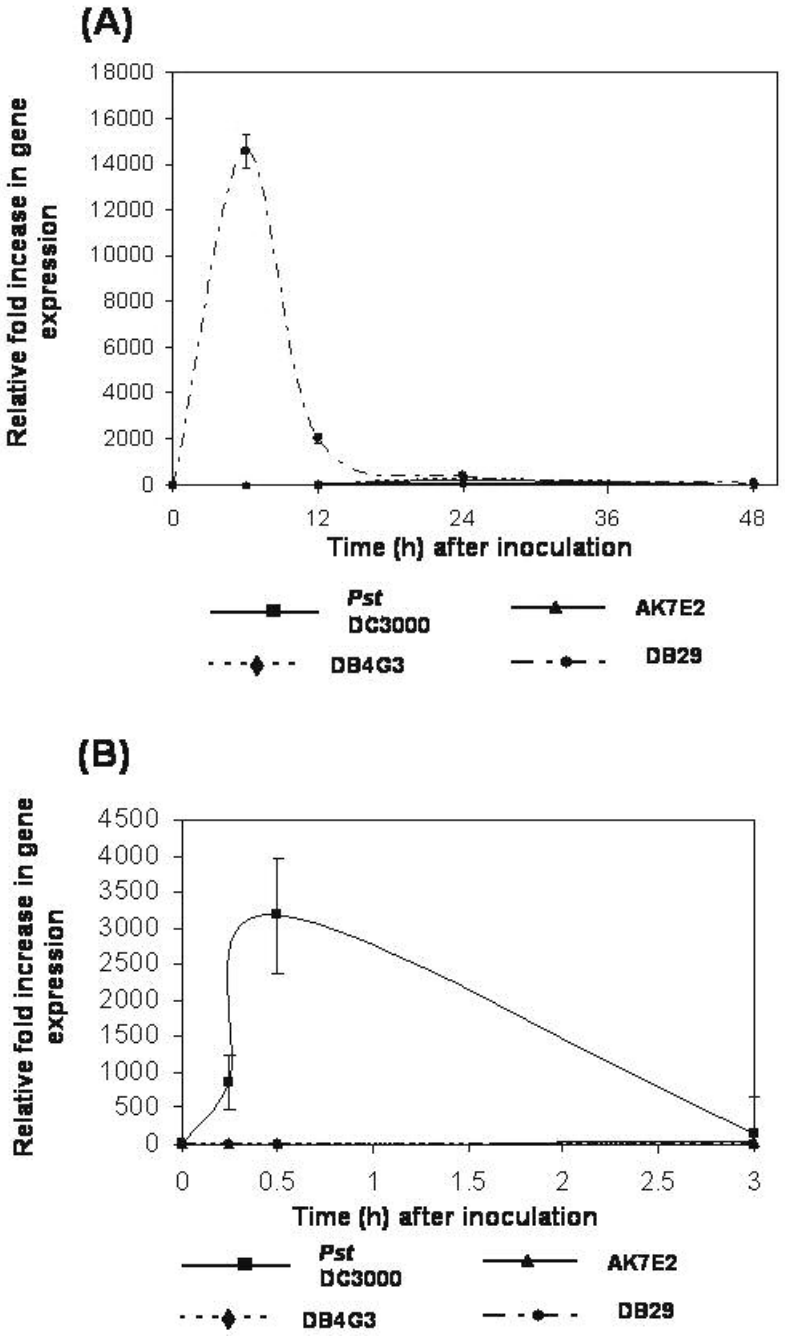
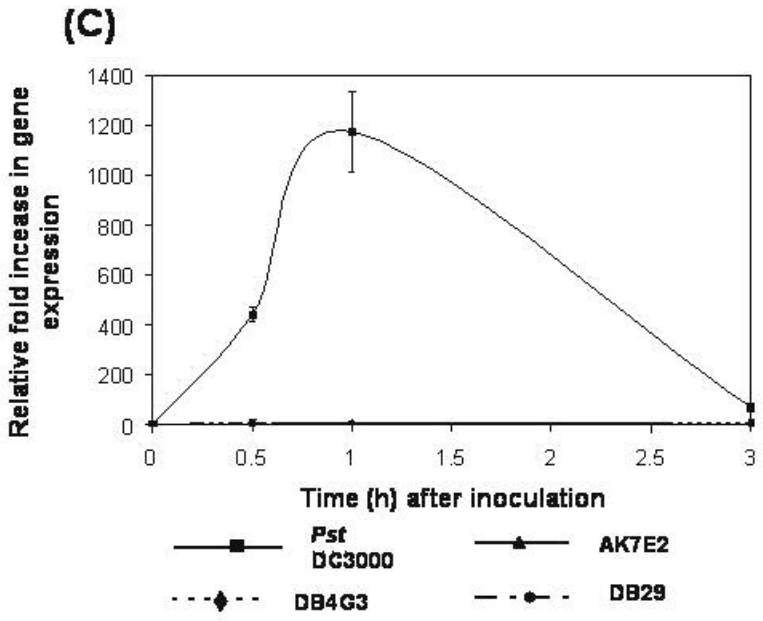
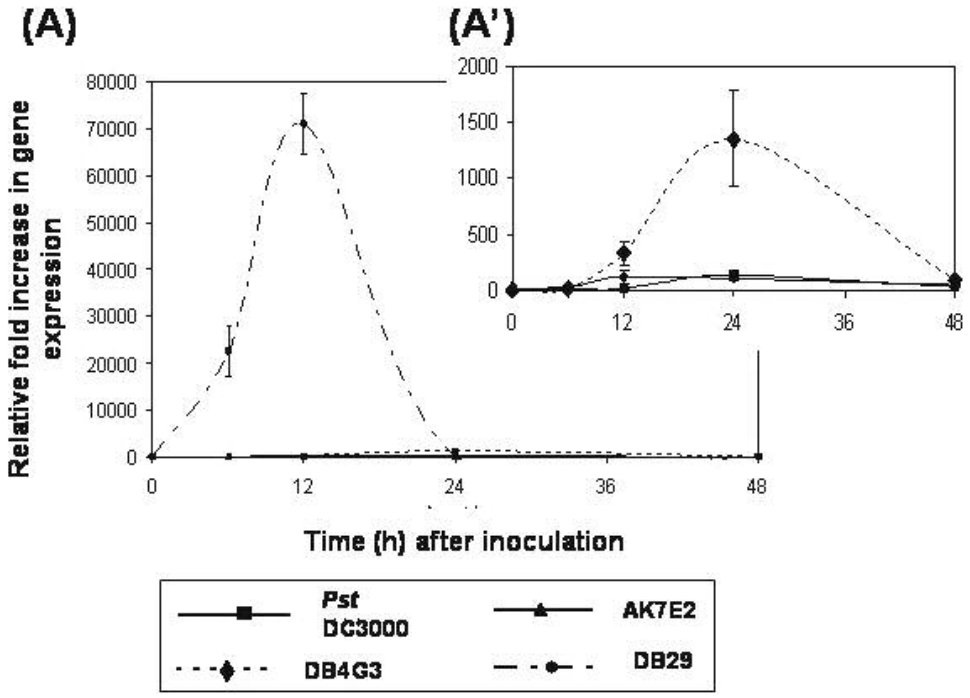


Fig.14. Analysis of gene expression in collard by RT-qPCR after inoculation with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA-cfa6* mutant). (**A**), *PR-1* expression at 0, 6, 12, 24 and 48 h; (**B**) *LOX2* gene expression at 0, 0.25, 0.5, 1, 2 and 3 h; and (**C**) *CORI1* gene expression at 0, 0.5, 1, 2 and 3 h. cDNA was synthesized from total RNA and real-time qPCR was performed using gene specific primers (Table 1).



In turnip, the kinetics of *PR-1* expression was slightly different than that observed in collard, with maximal expression occurring in leaves inoculated with the double mutant (DB29; *cmaA cfa6)* at 12 h (over 500-fold higher than the single mutants and *Pst* DC3000) after inoculation (Fig. 15A). When plotted independently of the DB29-treatment, *PR-1* expression was observed in turnip inoculated with the DB4G3 mutant (CFA^- CMA^+) at 24 h, and this expression was significantly higher (10-fold) than that in tissue inoculated with AK7E2 or *Pst* DC3000 (Fig. 15A'). This suggests that in turnip the whole toxin, as well as the CFA component alone, can suppress SA-associated defense. Maximal transcription of lipoxygenase (*LOX2*) was noted at 30 min after inoculation with *Pst* DC3000, while the level of transcription was approximately 120-fold lower when turnip was inoculated with the COR-defective mutants (Fig. 15B). There was a slight increase in the expression of *LOX2* gene (8-fold higher than with

DB29 and DB4G3) by AK7E2 (CMA^- CFA^+) at 30 min and 1h, suggesting that CFA (shown separately as in Fig. 15B') contributes partly to the modulation of JA pathways in turnip.



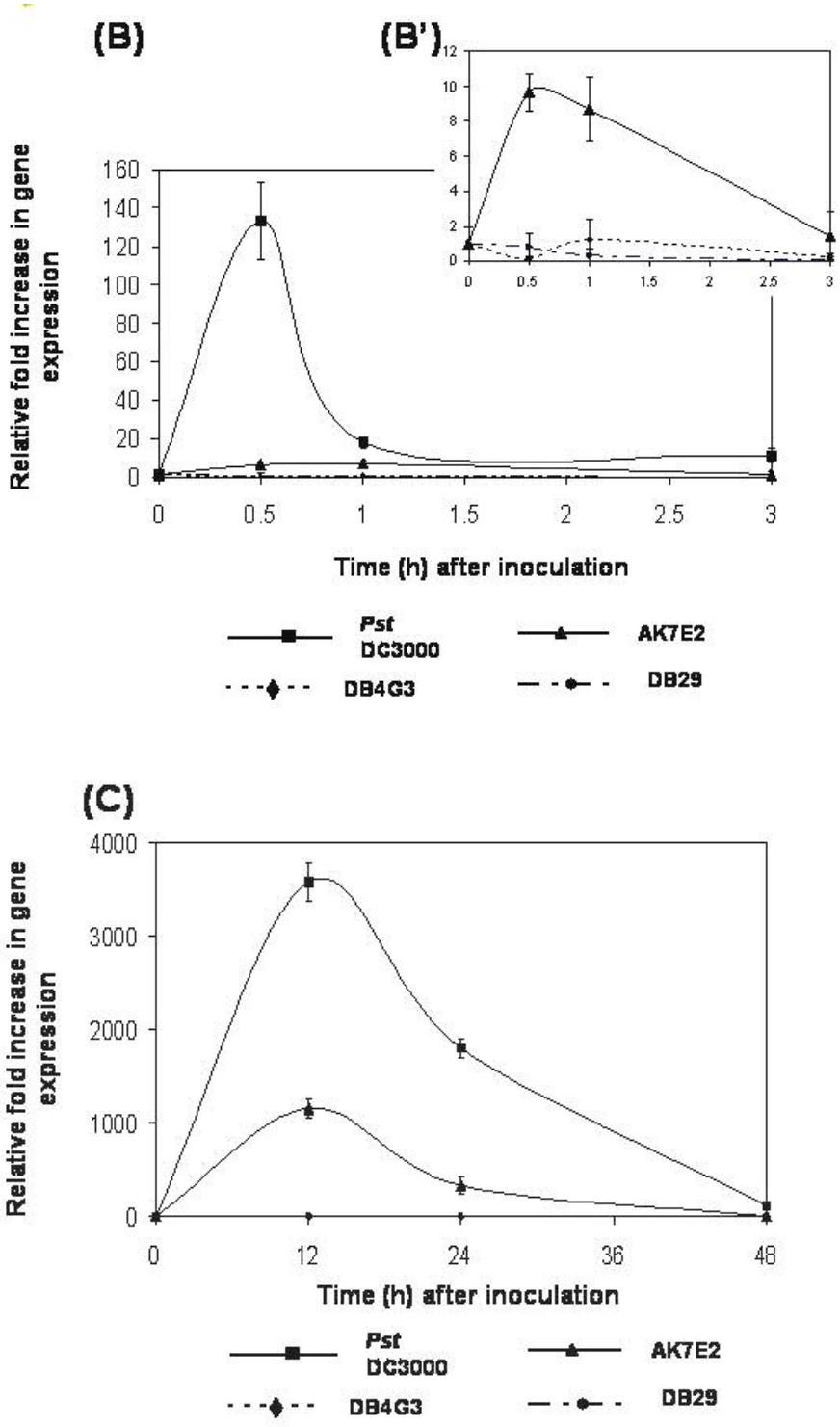


Fig.15. Analysis of gene expression in turnip by RT-qPCR after inoculation with *Pst* DC3000, AK7E2 (*cmaA* mutant), DB4G3 (*cfa6* mutant) and DB29 (*cmaA cfa6* mutant).

(**A**), *PR-1* expression at 0, 6, 12, 24 and 48 h; (**A'**), *PR-1* expression by *Pst* DC3000, AK7E2 and DB4G3 at 0, 6, 12, 24 and 48 h.

(**B**) *LOX2* gene expression at 0, 0.5, 1, 2 and 3 h; **(B')** *LOX2* gene expression in turnip treated with AK7E2, DB4G3 and DB29 at 0, 0.5, 1, 2 and 3 h.

(**C**) *CORI1* gene expression at 0, 6, 12, 24 and 48 h.

cDNA was synthesized from total RNA and real-time qPCR was performed using gene specific primers (Table 1).

Although chlorosis was not apparent until five days after inoculation of turnip with *Pst* DC000, *CORI1* (the first enzyme in chlorophyll degradation

pathway) was highly expressed at 12 h (over 600-fold higher than in plants inoculated with DB4G3 and DB29) after inoculation and then the expression dropped to a basal level after 48h (Fig. 15C). The expression of *CORI1* was also elevated in turnip plants inoculated with AK7E2 (CFA^+ CMA^-) at 12h after inoculation (Fig. 15C). The expression was 3-fold lower than that induced by *Pst* DC3000. These gene expression data agree with the chlorosis observed on turnip sprayed with *Pst* DC000 or AK7E2 (FIG. 7 A and B). *CORI1* gene expression was not observed in turnip plants inoculated with either DB4G3 or DB29.

The RT-qPCR expression analyses in the present study indicate that COR functions in part to suppress SA-associated defenses and to activation of the JA pathway in both collard and turnip. Furthermore, this study suggests that COR functions to degrade chlorophyll, an observation that has been reported by others in *A. thaliana* and tomato.

DISCUSSION

The results of this study indicate that collard and turnip respond very differently to infection with *Pst* DC3000 and the COR^- mutants. Symptom development in collard occurred much more rapidly than in turnip; for example, *Pst* DC3000-inoculated collard plants exhibited chlorotic, water-soaked lesions within three days post-inoculation. In contrast, symptoms were less severe on turnip and not apparent until five days after inoculation with *Pst* DC3000 (Table 3, Fig. 7). Unlike collard, turnip plants did not develop water-soaked lesions in

response to *Pst* DC3000, and anthocyanins did not accumulate in inoculated turnip leaves as they did in collard leaves.

COR-defective mutants were severely attenuated in virulence in comparison with *Pst* DC3000 (Fig. 6), and the lesion phenotypes in collard were similar for all three mutants (Table 2). In this respect, the results obtained with collard are similar to those reported for *A. thaliana*, in which the intact toxin is required for full manifestation of symptoms (Brooks *et al.*, 2004). Turnip plants inoculated with DB4G3 (*cfa6* mutant) and DB29 (*cmaA*-*cfa6* mutant) produced minor symptoms or none at all (Fig. 7C, D); however, when turnip was inoculated with AK7E2 (*cmaA* mutant), a substantial amount of chlorosis and necrosis developed (Fig. 7B). The symptoms observed in turnip plants inoculated with AK7E2 (*cmaA* mutant) may be due to the production of coronafacoyl conjugates, e.g. coronafacoylvaline, coronafacoylisoleucine, coronafacoylserine, and coronafacoylthreonine, which also induce chlorosis (Mitchell, 1984; Mitchell and Ford, 1998; Uppalapati *et al.*, 2005). Thus, the CFA moiety might form a conjugate with an amino acid present in turnip and the resulting coronafacoyl compound could induce the symptoms observed in turnip infected with AK7E2. These results indicate that the CFA component of COR is required for the bacterium to elicit disease symptoms in turnip.

COR contributes to the multiplication of *Pst* DC3000 in turnip

Although there was an obvious difference in the visual symptoms, the bacterial populations of *Pst* DC3000 and the COR-defective mutants were similar

in collard (Fig. 8). This finding differs from results reported for *A. thaliana*, in which all three COR mutants were compromised in their ability to multiply *in planta* (Brooks *et al.*, 2004). Thus in collard, COR contributes to disease symptom production, but not to multiplication *in planta*. In this respect, the results of this study are similar to those reported for Chinese cabbage inoculated with COR^+ and COR^- strains of *P. syringae* pv. *maculicola*, where COR contributed to the development of chlorosis but not to bacterial growth *in planta* (Tamura *et al.*, 1998).

In turnip, the bacterial populations of *Pst* DC3000 were higher than those of the COR^- mutants throughout the sampling period. Thus, in turnip, COR is important for both symptom development and multiplication of *Pst* DC3000, a finding that agrees with the results reported for tomato and *A. thaliana* (Brooks *et al.*, 2004; Uppalapati *et al.*, 2005). It is of significance that COR is thus shown to have two distinct roles in turnip, e.g. contributing to disease development and bacterial growth *in planta*.

Pst DC3000 alters pigment levels in collard and turnip

COR is a chlorosis-inducing phytotoxin, and a decrease in chlorophyll content was previously reported in tomato leaves inoculated with the toxin (Palmer and Bender, 1995). Ultrastructural studies demonstrated that chloroplasts in COR-inoculated tissue are significantly smaller than those in healthy tomato leaves (Palmer and Bender, 1995). In the present study, chlorophyll *a* and *b* levels in *Pst* DC3000-inoculated collard and turnip were only

20-30%, respectively, of those present in mock-inoculated leaves. Consistent with this finding, severe chlorosis was observed on both collard and turnip inoculated with *Pst* DC3000 (Figs. 6A, 7A). Furthermore, gene expression analysis (Figs. 14C, 15C) indicated that COR activates chlorophyllase, the principal enzyme in chlorophyll degradation. Thus, COR interferes with photosynthesis by degrading chlorophyll. A very early activation of *CORI1,* which encodes the first enzyme in the chlorophyll degradation pathway, in response to COR treatment has been shown previously in *A. thaliana* (Benedetti *et al*., 1998).

Anthocyanin accumulates in plants in response to many stresses including UV light, drought, extreme temperatures and pathogen infection (McClure, 1975). Foliar anthocyanin has been associated with plant resistance to herbivores and also has antioxidant activity (Coley and Kursar, 1996; Stone *et al.*, 2001; Wang *et al.*, 1997). The levels of anthocyanin were significantly higher in collard leaves inoculated with *Pst* DC3000 (COR^+) than in the leaves inoculated with COR^- mutants. Interestingly, anthocyanins did not accumulate to significant levels in turnip leaves, indicating a differential response in these two brassicas to *Pst* DC3000 infection. The high level of anthocyanin could be attributed to the early activation of the JA pathway, as JA is reported to induce the biosynthesis of anthocyanin in the wounded tissues of certain plants (Gould *et al.*, 2002; Richard *et al.*, 2000; Tamari *et al.*, 1995). Moreover, the expression of anthocyanin can be tissue and species-specific (Feys *et al*., 1994).

<u>COR suppresses SA-dependent defenses in both collard and turnip</u>

A popular hypothesis regarding the mode of action of COR is that the toxin promotes virulence by suppressing salicylic acid-associated defense in plants (Glazebrook *et al.*, 2003; Kloek *et al.*, 2001; Kunkel and Brooks, 2002). In the current study, this hypothesis was explored by analyzing the expression of *PR-1*, a gene that reflects activation of the SA pathway. Optimal expression of *PR-1* was observed in collard and turnip leaves 6 and 12 h, respectively, after inoculation with the *cmaA-cfa6* double mutant, DB29. In collard inoculated with *Pst* DC3000 and the single mutants (DB4G3 and AK7E2), *PR-1* expression was dramatically lower. However, DB4G3 (CFA^- CMA^+) induced a level of expression of *PR-1* in turnip, suggesting that COR and CFA (to a lesser extent) could suppress SA-dependent defenses in turnip. Thus COR, and possibly its components, function in part to suppress SA-dependent defenses, depending on the specific host-pathogen interactions. Previous studies in tomato also showed elevated *PR* gene expression in response to inoculation with a COR^- mutant when compared to that following inoculation with *Pst* DC3000 (Zhao *et al.*, 2003).

COR stimulates JA-mediated signaling in brassicas

Several prior investigations have confirmed the activation of JA-mediated responses by COR in *A. thaliana* and tomato (Block *et al.*, 2005; Feys *et al.*, 1994; Schmelz *et al.*, 2003; Uppalapati *et al.*, 2005). In this study, the lipoxygenase gene, *LOX2*, was expressed very early in collard and turnip after inoculation with *Pst* DC3000. Although there is a difference in the timing of disease symptom development in collard and turnip, the JA pathway is activated

very early in both brassicas. Microarray analysis has shown early activation of *LOX2* genes in *A. thaliana* (Reymond *et al*., 2000). These findings support the theory that COR acts as a JA analog, which then suppresses SA-associated host defense, thus enhancing pathogen virulence (Kloek *et al.*, 2001; Mittal and Davis, 1995). Interestingly, a slight activation of the *LOX2* gene was observed in turnip treated with AK7E2 (CMA^- CFA^+). This increase could be due to the structural and functional similarity of CFA and COR to JA, which in turn might be significant in the interaction with turnip and not with collard. It has been argued that COR-mediated virulence occurs via SA-dependent and SA-independent mechanisms (Block *et al.*, 2005; Feys *et al.*, 1994; Schmelz *et al.*, 2003; Uppalapati *et al.*, 2005)). Our results show that *PR-1* expression is suppressed by COR and by one or both of its components. The expression of *PR-1* in collard and turnip inoculated with COR^- bacteria suggests that COR modulates virulence at least partly through a SA-dependent mechanism. The early activation of jasmonate-mediated responses when COR is produced may contribute to the suppression of SA-mediated defenses in brassicas. To our knowledge, this is the first study conducted to characterize the molecular interactions of *Pst* DC3000 with edible species of brassicas and to monitor defense gene expression in these hosts using real-time qPCR.

CHAPTER IV

The Response of Edible Brassicas to Exogenous Application of Coronatine and its Components, Coronafacic and Coronamic Acid

SUMMARY

The phytotoxin coronatine (COR), which is produced by several pathovars of *P. syringae*, causes a wide variety of biological responses in plants. In the present study, we studied the effect of COR and its components, coronafacic acid (CFA) and coronamic acid (CMA), on collard and turnip, which are *Brassica* spp. grown in Oklahoma. Both collard and turnip were treated with 0.2 nmol each of CFA, CMA and COR. The toxin induced chlorophyll degradation and chlorosis on both collard and turnip, but the response of turnip was later than that in collard. Gene expression analysis revealed that COR activated the gene encoding chlorophyllase (*CORI1*) in both *Brassica* spp. *LOX2*, which indicates activity in the jasmonic acid pathway, was expressed very early in both plant species treated with COR. The application of exogenous CFA also promoted *LOX2* expression in turnip, but this effect was not observed in collard. RT-qPCR analysis suggested that the CFA component of COR may be partly accountable for JA-responsive defense gene expression in turnip, but not in collard. *PR-1*, a gene used to monitor activity in the pathway leading to production of salicylic acid

(SA), was not induced by the application of COR, CFA, or CMA. Altogether, there were dissimilarities between collard and turnip in the time and intensity of chlorosis development as well as the relative fold induction of different marker genes. Moreover, the phenotypic responses and gene expression of the two plant species after exogenous COR and related compounds were applied were considerably different from those observed after inoculating the plants with COR^+ and COR^- bacterial strains. These results suggest that the bacterial strain also plays an important role in determining the response of host plants to COR and its components.

INTRODUCTION

Coronatine (COR), a non host-specific phytotoxin produced by certain pathovars of *Pseudomonas syringae*, functions as a virulence factor in a number of *P. syringae*-host plant interactions (Bender *et al.*, 1999; Brooks *et al.*, 2004; Penaloza-Vazquez *et al.*, 2000). COR induces a range of effects in plants such as chlorosis, hypertrophy, root inhibition, ethylene production and accumulation of anthocyanin and proteinase inhibitors. The major biological effect on leaves treated with COR is the development of intense chlorosis, which has been ascribed to the rapid break down of chlorophyll in the plant (Kenyon and Turner, 1992; Palmer and Bender, 1995; Uppalapati *et al.*, 2005). In *A. thaliana* as well as tomato, COR stimulated the expression of chlorophyllase, the first enzyme in

the chlorophyll degradation pathway (Benedetti and Arruda, 2002; Brooks *et al.*, 2005 in press; Tsuchiya *et al.*, 1999).

The COR molecule is composed of the polyketide coronafacic acid (CFA) and coronamic acid (CMA), which is a cyclized derivative of isoleucine; these two parts are linked together by an amide bond. COR functions in part as a structural and functional analog of jasmonic acid (JA) and endogenous jasmonates in *A. thaliana* and tomato (Feys *et al.*, 1994; Zhao *et al.*, 2003). It has been speculated that COR contributes to virulence by functioning as a JA analog, and that induction of the JA pathway occurs at the expense of salicylic acid (SA)-mediated defenses (Block *et al.*, 2005; Zhao *et al.*, 2003). The SA pathway was previously shown to be a critical component of a successful defense response in the *A. thaliana-P. syringae* interaction (Delaney *et al.*, 1994), and COR was reported to suppress SA-related plant defenses in *A. thaliana* inoculated with *P. syringae* (Brooks *et al*., *in press*; Kloek *et al*., 2001). The expression of the *LOX2* and *PR-1* genes are markers that are utilized to follow the activation of JA- and SA-associated defense responses, respectively (Creelman and Mulpuri, 2002; Farmer *et al.*, 2003; Glazebrook, 2001).

Results obtained with *A. thaliana* and tomato have been invaluable in furthering our understanding of the role of COR and its components. However, it is important not to overextend our interpretation of the function of COR and related molecules based on observations with only two plant species. Furthermore, the response of plants treated with the purified compounds may differ considerably from those obtained when plants are inoculated with the COR-

producing pathogen. In the present study, we treated both collard and turnip leaves with equimolar concentrations of COR, CFA and CMA and evaluated the phenotypic response, pigment production, and expression of genes encoding chlorophyllase (*CORI1)*, *LOX2*, and *PR1*. The results show that COR induces chlorophyll degradation and subsequent chlorosis in both the hosts. Although COR activated JA responses and chlorophyll degradation in both collard and turnip, there were distinct differences in the timing and manifestation of chlorosis and considerable variation in the relative fold increase in gene expression in the two *Brassica* spp.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of collard (*Brassica oleraceae* var. *viridis* L. cv. Vates) and turnip (*B. rapa* var. *utilis* (DC) Metzg. cv. Alltop) were obtained from Twilley Seed Co. (Hodges, SC). The plants were grown in Metro-Mix® 200 (Sierra Horticultural Products Co., Marysville, OH) and maintained in a growth room at 24-25^{0}C, 35-40% relative humidity (RH), and a photoperiod of 12 h. COR, CFA and CMA were applied as droplets of 0.2nmol onto the leaves of four-week old plants.

Exogenous application of COR, CFA and CMA

The leaves of collard and turnip plants were treated with equimolar concentrations (0.2 nmol) of purified COR and its components, CFA and CMA.

Stock solutions (0.1 mg/ml) of each compound were maintained in methanol and stored at 4^0C. Aliquots of each compound were removed from the stock solutions, and methanol was removed by evaporation in a water bath maintained at 50 to 55^0C. The compounds were then suspended in water and applied to turnip or collard leaves in 2 µL droplets (63.8, 41.6 and 25.8 ng of COR, CFA and CMA, respectively and equivalent to 0.2nmol). Treated plants were then transferred to a growth chamber maintained at 25^0C, 12h photoperiod and 70% relative humidity.

Symptoms and pigment estimation

Treated plants were monitored for disease symptoms during a seven day period. The extent of chlorosis and pigment changes in plants treated with COR were noted and compared to those of CFA, CMA and mock-treated (sterile distilled water) plants. The quantity of chlorophyll and anthocyanin in treated leaves was estimated seven days after inoculation. Leaf discs (10 mm diameter) were macerated in ice-cold ethyl acetate/acetone (1:1 v/v). This suspension was diluted (1:33 ratio) in the same solvent and absorbance was read at 665 and 649 nm to estimate chlorophylls *a* and *b*, respectively. Leaf discs of the same size were agitated gently in 1 ml of a solution containing 3M HCl: H_2O: methanol (1:3:16 volume ratio) in the dark for 24 h at 4^oC. The absorbance was read at 530 and 653 nm, and anthocyanin levels were estimated as A_{530}-0.24 $A_{653.}$ The deduction of 0.24 A_{653} compensates for a small overlap in absorbance at A_{530} by

chlorophyll *a* and *b*. The readings were plotted as percentage chlorophyll or anthocyanin present in treated leaves.

Northern blot analysis

Total RNA was extracted from inoculated leaves collected at different time points (0, 12, 24 and 48 h) using TRIZOL reagent, a mono-phasic solution of phenol and guanidine isothiocyanate (Sigma). During sample (100 mg of leaf tissue) homogenization, TRIZOL disrupts cells and dissolves cell components, while maintaining the integrity of the RNA. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains entirely in the aqueous phase, which is recovered by precipitation with isopropyl alcohol.

Ten µg of RNA (extracted as described above) was electrophoresed on formaldehyde gels, transferred to nylon membranes, and fixed to membranes using UV light (Stratalinker® UV Crosslinker). Hybridization probes were prepared using the Random Primer Labeling kit (Invitrogen Life Technologies, Carlsbad, CA). *A. thaliana* cDNA clones containing genes indicative of the SA pathway (*PR-1*), the JA pathway (*LOX2*) and chlorophyllase (*CORI1*) were used as probes. The probes were received from the lab of Dr. Barbara N. Kunkel (Washington University, St. Louis, MO). The *LOX2* probe was a 1.0 kb *Eco*RI-*Bam*HI cDNA fragment from *A. thaliana LOX2* (*AtLOX2*) gene and was cloned into pZL1 (Brooks *et al*., *in press*). The *AtLOX2* gene was cloned and characterized (Genbank Accession No. L23968) by Bell and Mullet (1993). The *PR-1* probe contained a 750 bp *Eco*RI-*Xho*I fragment with the complete coding

sequence of the *A. thaliana PR-1* gene (Genbank Accession No. NM_127025.2) (Brooks *et al.*, *in press;* Uknes *et al.*, 1992). The *CORI1* probe contained a 1.15 kb *Eco*RI-*Xho*I fragment with the complete coding sequence of *A. thaliana CORI1* gene (Genbank accession number- AF021244) (Benedetti *et al.*, 1998; Brooks *et al.*, 2005 in press). The cDNA (50 ng) was labeled with ^{32}P (50 µCi), hybridized at 45^{o}C overnight, and washed twice (2X SSC at room temperature and 1X SSC at 45^{o}C). RNA blots were exposed for 3 to 4 days and analyzed using a phosphorimager.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from leaves inoculated with *Pst* DC3000 and the COR^{-} mutants at 0, 0.5, 1, 3, 6, 12, 24 and 48 h after inoculation. The quantity of RNA was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by gel electrophoresis. The extracted RNA was treated with 1 µL (1 U per µL) of amplification grade DNAse I (Invitrogen) to avoid contamination by genomic DNA. DNAse I was inactivated by adding 1 µL of 25 mM EDTA and incubating at 65^{o}C. 1 µg of the DNAse-treated RNA was used to synthesize cDNA using the Superscript ^{TM} First Strand Synthesis System (Invitrogen). RNA not incubated with reverse transcriptase was included as a negative control in the unlikely event that samples contained any remaining genomic DNA. The quantity of cDNA was estimated using the NanoDrop ND-1000 spectrophotometer, and the volume was adjusted to 100 ng per reaction.

Primers for *PR-1*, *LOX2*, *CORI1* and 18S rRNA (included as a constitutively expressed control) were designed using D-LUX™ Designer software available from Invitrogen (www.invitrogen.com/lux). The primer sets were predicted to amplify fragments ranging in size from 60 to 105 bp (see Chapter III, Table 1). One primer from each set was labeled with the fluorophore JOE (6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein). All the primers were designed from regions of Arabidopsis genes showing the highest homology with *Brassica* spp. The sequences of *PR-1*, *LOX2*, *CORI1* and 18S rRNA from *A. thaliana* were aligned with available ESTs of *Brassica* spp. The Basic Local Alignment Search Tool (BLAST) from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find regions of similarity between sequences.

PCR was performed using the HotStarTaq Master Mix Kit (250 U Kit; Qiagen Inc., Valencia, CA), which included HotStarTaq DNA polymerase (250 U), $MgCl_2$ (final reaction concentration 1.5 mM), and 200 μM of each dNTP. The total reaction volume for real-time PCR was 25 μl, and each reaction contained 3 μM of the gene specific primers (1 μl), 12.5 μl of the HotStar Taq Master Mix, and 100 ng of the cDNA template (2 μl). The reactions were incubated at 95°C for 15 min, and then cycled (40 X) at 95°C for 15 s, 60°C for 1 min and 72°C for 34 s. Reactions were performed in a 96-well format in a spectrofluorometric thermal cycler (ABI PRISM 7700 Sequence Detector System, Applied Biosystems). The amount of fluorescence as a function of PCR cycle was plotted using the ABI PRISM 7700 SDS software, and the threshold cycle (C_t)

values were obtained. The C_t values for all target genes were first normalized using the gene encoding 18S rRNA to get ΔC_t. Gene expression was calculated by the comparative C_t method, where the relative amount of target gene expression is compared to the gene expression at time zero (calibrator) to get the $\Delta\Delta C_t$. Target gene expression was represented as the relative fold increase in the transcript level with reference to gene expression at time zero. The relative differences in target genes were calculated according to the $\Delta\Delta C_t$ mathematical model (Pfaffl, 2001).

RESULTS

Symptoms on collard and turnip treated with COR, CFA and CMA

The effects of purified COR and its constituents were studied by the exogenous application of COR, CFA and CMA onto the leaves of collard and turnip. Chlorotic symptoms were first apparent on collard leaves on the third day following application of COR (Fig. 16D). Symptoms appeared as round, chlorotic regions (5 to 10 mm diameter) surrounding the region where COR was applied. The application of CFA and CMA did not induce any visible symptoms and were indistinguishable from those of the water-inoculated control.

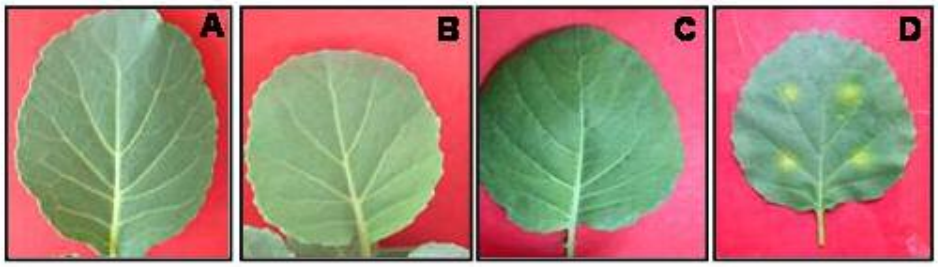


Fig.16. Symptoms on collard leaves treated with: (A) water control; (B) CFA; (C) CMA; and (D) COR. The leaves were inoculated with 0.2 nmol of the purified compounds and photographed seven days after treatment.

Chlorotic symptoms were visible on turnip beginning five days after treatment. In this host, the chlorosis had a diffuse pattern and was less intense (Fig. 17D) than that observed in collard (Fig. 16D). No symptoms were induced by CFA or CMA. These results indicate that the intact COR toxin, when applied as a purified compound in the absence of the bacterium, is required for chlorosis in both *Brassica* spp.

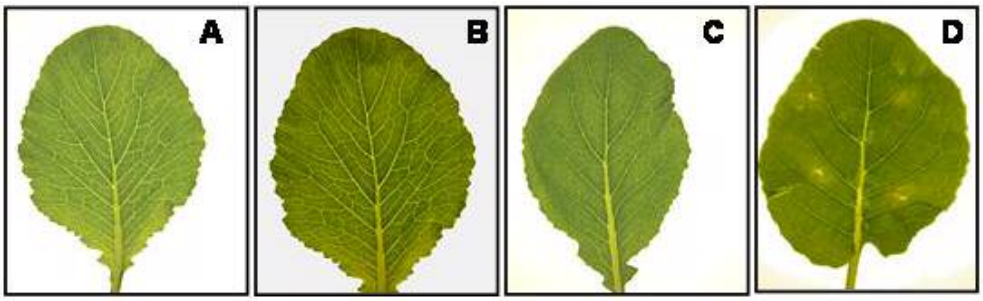


Fig.17. Symptoms on turnip leaves treated with: (A) water control; (B) CFA; (C) CMA; and (D) COR. The leaves were inoculated with 0.2 nmol of the purified compounds and photographed seven days after treatment.

Chlorophyll and anthocyanin estimation

The chlorophyll content of COR-treated collard leaves was approximately 30% of that observed in healthy, water-inoculated control tissue (Fig. 18). These findings agree with the observation of leaf chlorosis. CFA and CMA treated tissues showed a slight reduction in chlorophyll *a* and *b* levels, but the reduction was not enough to result in visible symptoms and was not statistically significant (Fig. 16B, C). The levels of anthocyanin pigment did not change in any of the CFA, CMA and COR treated collard leaves (data not shown). This result is consistent with the lack of noticeable anthocyanin production on treated leaves.

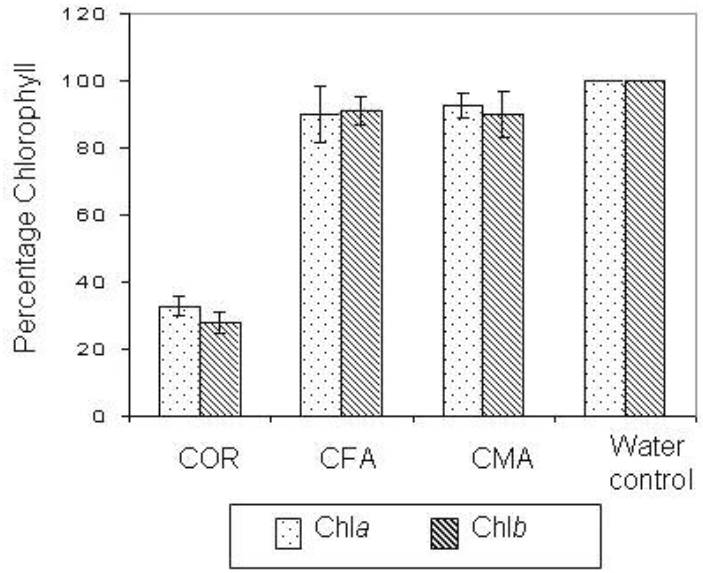


Fig.18. Chlorophyll content in collard leaves treated with COR, CFA, CMA and water. The leaves were inoculated with 0.2 nmol of the purified compounds. The pigment level was analyzed 7 days after inoculation, and the analysis was repeated two times with similar results.

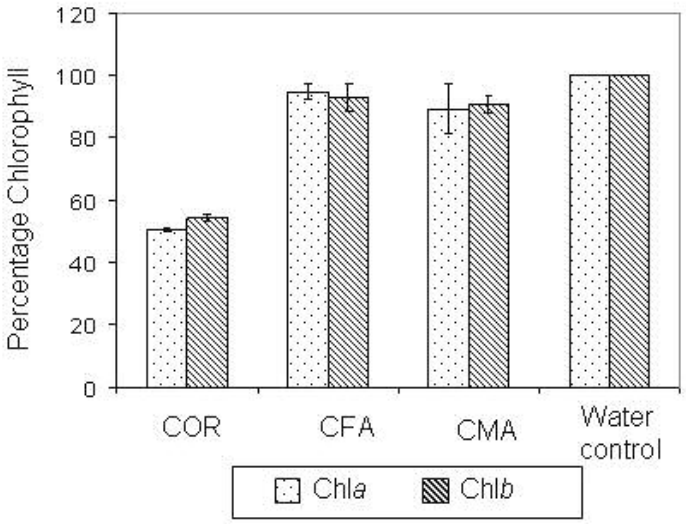


Fig.19. Chlorophyll content in turnip leaves treated with COR, CFA, CMA and water. The leaves were inoculated with 0.2 nmol of the purified compounds. The pigment level was analyzed 7 days after inoculation, and the analysis was repeated two times with similar results.

The level of chlorophyll in turnip leaves treated with COR was approximately 50% of that in leaves receiving control treatment, whereas treatment with CFA and CMA did not result in a significant reduction in pigment levels (Fig. 19). The anthocyanin levels in all treatments were equivalent to that of the water-inoculated control (data not shown). This finding suggests that COR and its components are unable to stimulate anthocyanin production in turnip leaves.

Gene expression analysis

The gene that encodes lipoxygenase (*LOX2*) was expressed at all the time points of analysis in collard leaves treated with CFA, CMA and COR (Fig. 20A). *CORI1*, which encodes chlorophyllase, was expressed at 24 and 48 h (visible as weak signals) after treating collard leaves with COR (Fig. 20B). This result is in agreement with the manifestation of chlorosis induced in collard by COR. However, the application of CFA and CMA did not induce the expression of *CORI1*.

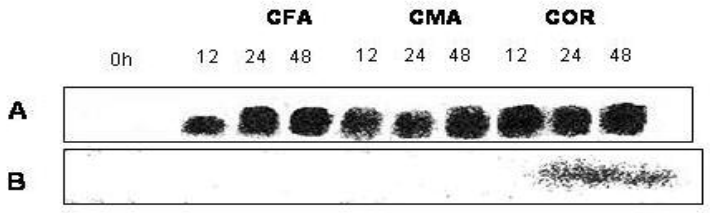


Fig.20. Gene expression in collard treated with CFA, CMA and COR by RNA blot analysis. Expression of (**A**) *LOX2* and (**B**) *CORI1* at 0, 12, 24 and 48 h after treatment with 0.2 nmol of each CFA, CMA and COR.

In turnip leaves, application of CFA, CMA and COR induced *LOX2* expression, which was apparent at 12 h and at each time point thereafter. A weak expression of *CORI1* was noticed in turnip leaves, but only 48 h after COR treatment (Fig. 21B). The treatments with CFA and CMA did not induce the expression of *CORI1* in turnip.

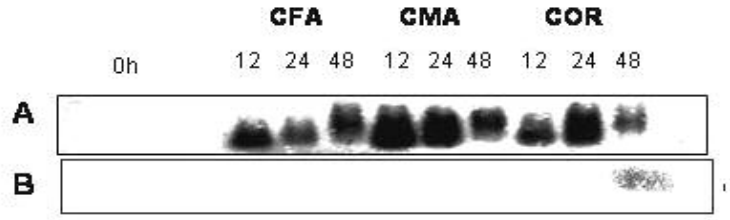


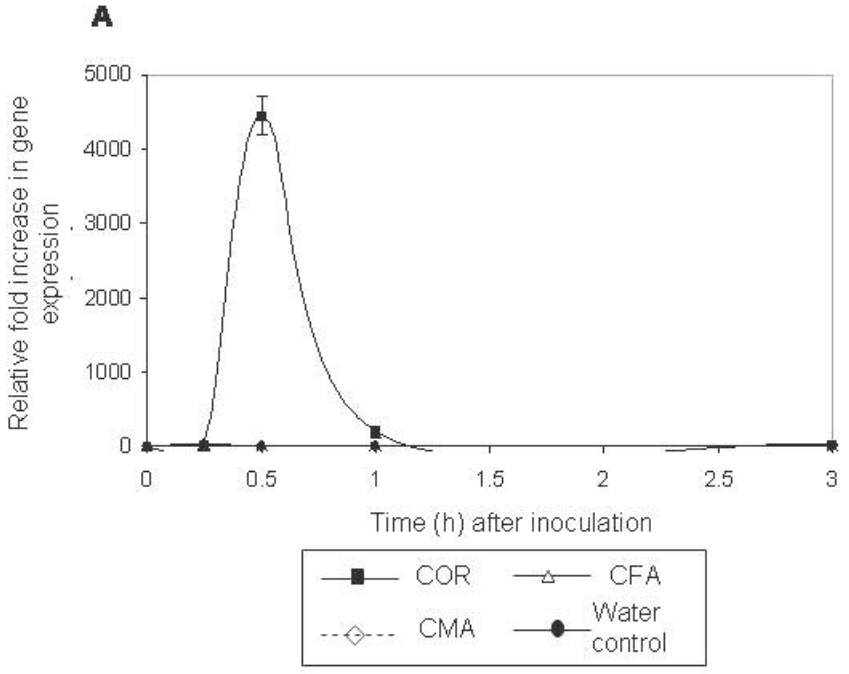
Fig.21. Gene expression in turnip treated with CFA, CMA and COR by RNA blot analysis. Expression of (**A**) *LOX2* and (**B**) *CORI1* at 0, 12, 24 and 48 h after treatment with 0.2 nmol of CFA, CMA and COR.

Sensitivity limitations in the RNA blots were caused by the use of very large probes that were derived from *A. thaliana* in northern blots, and lacked 100% homology to sequences in the *Brassica* spp. To achieve greater sensitivity, RT-qPCR analysis was used to analyze expression of *LOX2, CORI1* and *PR-1* in collard and turnip leaves treated with COR, CFA, and CMA. In addition to the time points used for northern blots (0, 12, 24 and 48 h post inoculation), early time points including 0.5, 1, 3, 6 h were also included to monitor the relative-fold increase in gene expression. In the case of *LOX2*, expression was also analyzed at 0.25 h.

An increase in *LOX2* expression was observed in collard leaves 30 min after treatment with COR (Fig. 22A). The increase in gene expression was over

200-fold higher in COR-treated tissue than in CFA, CMA and mock-inoculated controls (Fig. 22A). These results suggest that the JA pathway is activated very quickly after purified COR is applied to collard leaves.

In collard leaves treated with COR, an increase in *CORI1* expression was apparent 6-12 h after treatment with exogenous COR. Maximal expression, observed 6 h post treatment, was 35-fold higher than in the mock-inoculated control or the CFA and CMA treatments (Fig. 22B) These observations are consistent with the chlorosis visible on COR-treated leaves and the reduction in chlorophylls *a* and *b* (Figs. 16 and 18, respectively).



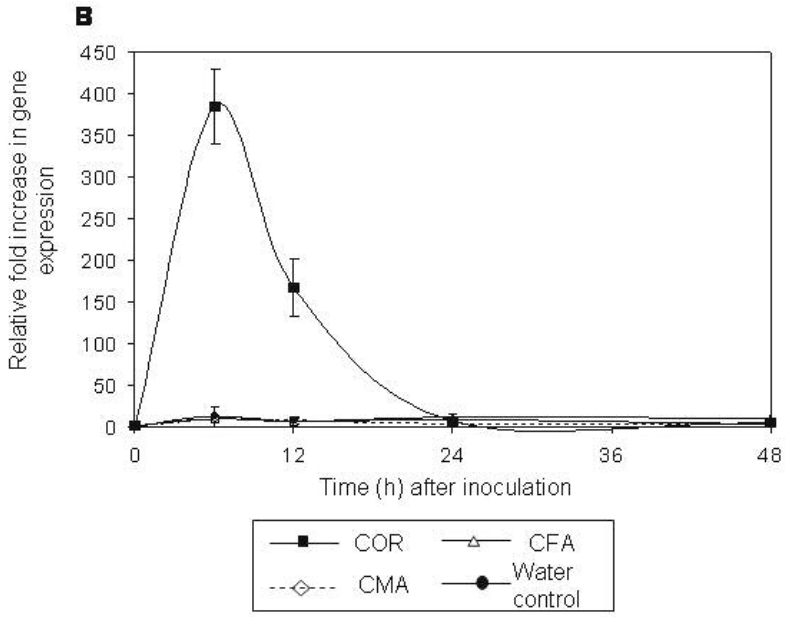
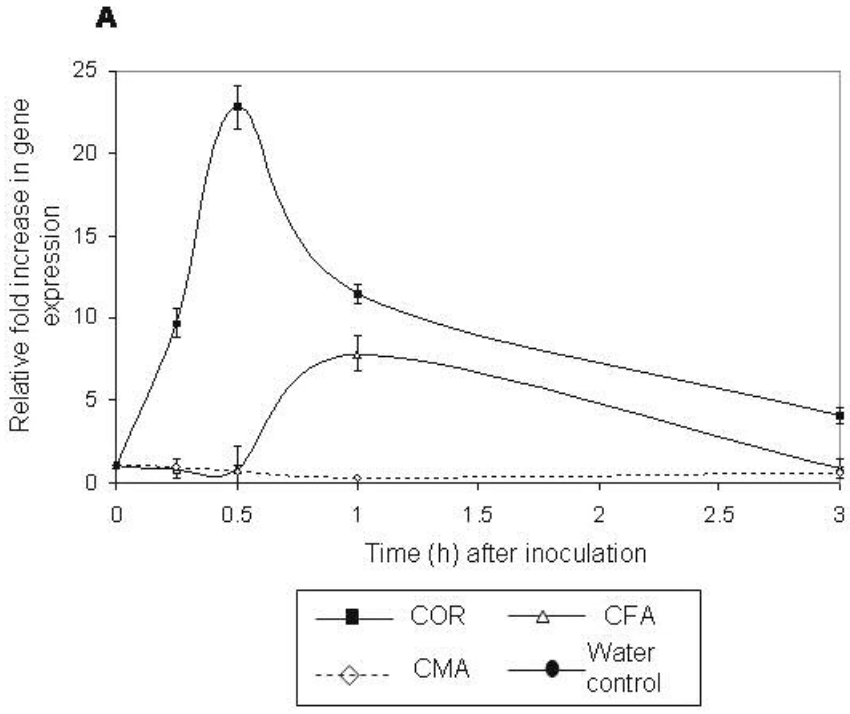


Fig.22. Analysis of gene expression in collard by real-time qPCR after treatment with 0.2 nmol CFA, CMA or COR. **A**, Relative-fold increase in *LOX2* expression at 0, 0.5, 1, and 3 h after treatment. **B**, *CORI1* expression at 0, 3, 6, 12, and 24 h after treatment. cDNA was synthesized from total RNA and real-time qPCR was performed using gene specific primers (Chapter III, Table. 1.)

In turnip, JA-associated *LOX2* gene expression showed a maximal relative-fold increase 30 min after inoculation with COR. The relative-fold increase in *LOX2* expression in COR-treated tissue at 30 min was 43, 28 and 34-fold higher than that observed in turnip leaves treated with sterile water (mock-treatment), CFA, or CMA, respectively. Interestingly, there was a significant relative-fold induction of *LOX2* apparent 1 h after treatment with CFA (Fig. 23A). There was a 30-fold difference in the relative-fold induction of *LOX2* expression in response to CFA as compared to CMA expression at the 1 h time point. However, *LOX2* induction at 1h was still 1.4 fold higher in COR treated leaves than was the CFA induced expression. These results suggest a potential role of CFA in modulating the *LOX2*-mediated JA responses in turnip. In turnip, *LOX2*

expression is triggered by both CFA and COR, probably due to the structural similarity of these compounds to JA. Other than *LOX2* induction, CFA does not induce any other response in turnip.

The chlorophyllase-encoding gene, *CORI1,* showed a high relative-fold increase in gene expression in COR-treated turnip leaves beginning at 12 h; this increase was transient, with a slight change in relative-fold induction detected at 24 h (Fig. 23B). The relative-fold increase in *CORI1* expression was over 120-fold higher in COR-treated turnip tissue at 12 h as compared to other treatments. Thus, the expression of *CORI1* obtained by real-time qPCR agrees with the low chlorophyll levels observed in turnip leaves treated with COR, but not with CFA or CMA (Figs. 17 and 19).



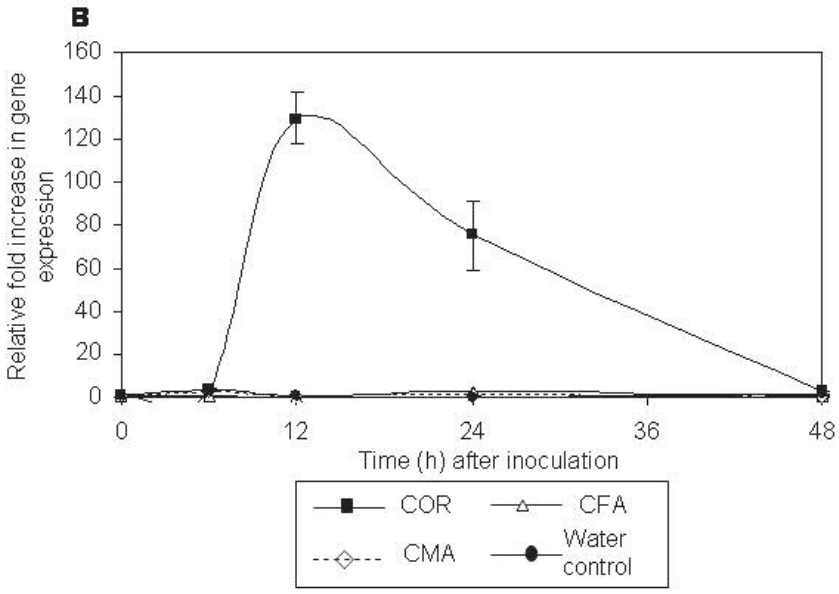


Fig.23. Analysis of gene expression in turnip by real-time qPCR after treatment with 0.2 nmol CFA, CMA or COR. Relative-fold induction of: **A**, *LOX2* at 0, 0.5, 1, and 3 h; and **B**, *CORI1* expression at 0, 6, 12, 24, and 48 h. cDNA was synthesized from total RNA and real-time qPCR was performed using gene specific primers (Chapter III, Table. 1.)

There was no expression of the SA-pathway marker gene, *PR-1*, in collard or turnip treated with COR, CFA, CMA, or mock-inoculation with water. It is likely that COR and its components either suppress the SA-associated defense in both the host plants or may have no effect on induction of the SA pathway in the absence of the bacteria. The data available from the present study is not enough to draw any conclusions on the effect of purified CFA, CMA or COR on SA-mediated defense responses in collard and turnip. A feasible experiment to resolve this issue is to induce the expression of *PR-1* gene followed by the application of these compounds to monitor their effects on expression/suppression of the *PR-1* gene *in planta*. This would be done by spray

inoculating the plants with salicylic acid followed by the treatment of both plants with CFA, CMA and COR. The *PR-1* levels in the plants will be lowered if any of the compounds suppressed SA pathway. These experiments would confirm the likely effects of COR and its components on SA mediated defense responses are in progress.

DISCUSSION

Both collard and turnip plants developed chlorosis when treated exogenously with 0.2 nmol purified COR, but showed no other visible foliar symptoms. In collard, COR induced round, chlorotic regions, which were apparent 3 days post-treatment; however, the chlorosis on turnip was delayed (not apparent until 5 days), and the chlorotic regions were more diffuse and less localized than those observed in collard leaves (Figs. 16 and 17). The appearance and timing of chlorosis in turnip was comparable to those observed in tomato, where moderate to severe yellowing was apparent five to eight days after COR treatment (Palmer and Bender, 1995; Uppalapati *et al.*, 2005). The differences in the timing and pattern of chlorosis between host plant species could be due to the difference in perception of COR by putative receptors in individual host plants. In both collard and turnip, the intact COR toxin was required to induce chlorosis; in this respect, these results agree with those obtained in *A. thaliana* and tomato (Brooks *et al.*, 2004; Uppalapati *et al.*, 2005).

It is important to compare the results obtained with exogenous compounds to those obtained with the defined COR-defective mutants (Chapter III). For

example, although the (CFA^+ CMA^-) mutant AK7E2 induced chlorotic lesions on turnip, (Chapter III, Fig. 7B) the exogenous application of CFA did not induce chlorosis. This difference might be due to the inability of exogenous CFA to form chlorosis-inducing coronafacoyl conjugates, possibly because of the absence of coronafacoyl ligase, an enzyme that may be present only in the bacterium.

Another interesting difference in the results obtained with exogenous compounds was the absence of anthocyanin pigments in collard leaves treated with purified COR. This contrasts with the high levels of anthocyanin that accumulated in collard leaves spray-inoculated with the COR-producing *Pst* DC3000 (Chapter III, Fig. 6D). This finding suggests that anthocyanin accumulation is a product of the host-bacterium interaction, and application of exogenous COR alone is not sufficient to induce anthocyanins. Similar results were observed in tomato, in which leaves inoculated with COR did not accumulate anthocyanins (Uppalapati *et al.*, 2005).

It has been reported that COR enhances the loss of chlorophyll in various plant species (Kenyon and Turner, 1992; Palmer and Bender, 1995; Uppalapati *et al.*, 2005). In the present study, the level of chlorophyll *a* and *b* in collard were reduced to 30% in COR-treated collard leaves and were 50% lower in COR-treated turnip leaves than in the mock-inoculated leaves (Figs. 18 and 19). No difference in the chlorophyll level was observed on either CFA or CMA-treated leaves of collard or turnip. Furthermore, chlorosis was observed on collard and turnip leaves treated with COR, but not those treated with CFA or CMA (Figs. 16 and 17). Ultra structural and immunolocalization studies previously suggested the

association of COR with the chloroplasts (Palmer and Bender, 1995; Zhao *et al.*, 2001). Gene expression analysis by RNA blot analysis and RT-qPCR showed the activation of the chlorophyllase-encoding gene, *CORI1*, in the COR-treated leaves of both collard and turnip. The expression of *CORI1* by COR was previously reported (Benedetti and Arruda, 2002; Brooks *et al*., *in press*; Tsuchiya *et al*., 1999). *CORI1* gene expression, as analyzed by RT-qPCR was activated at an earlier time point in collard than in it was in turnip, and this result supports the conclusion that chlorosis develops earlier in collard. These results imply that COR may down-regulate genes associated with the photosynthetic apparatus.

Many previous reports suggest that COR functions as a molecular mimic of JA and associated molecules (Block *et al.*, 2005; Feys *et al.*, 1994). The *LOX2* gene, whose expression is indicative of activity in the JA pathway, was induced very quickly after treatment of collard and turnip with COR (within 30 min). In turnip, there was also a significant increase in *LOX2* gene expression 1 h after treatment with CFA, but the relative-fold increase in gene expression was 1.4-fold less than that observed with COR. This observation suggests that the CFA component has a potential role in modulating JA-mediated defenses in turnip. Similar results were observed for lipoxygenase gene expression in tomato leaves treated with CFA and COR (Uppalapati *et al.*, 2005). For example, the CFA moiety regulated 40% of the COR-modulated genes and most of the JA-responsive genes (Uppalapati *et al.*, 2005). However, it is important to note that

tissue-specific and species-specific differences can exist and broad interpretations based on a few studies may not be valid (Feys *et al.*, 1994) .

The expression of *PR-1*, which is a marker indicative of activity in the SA pathway, was not observed in turnip or collard treated with COR, CFA, or CMA. It is not clear from the available data if SA-mediated defense responses are suppressed or not influenced by these compounds. The cross-talk of COR with other virulence factors in the pathogen (like Type III effectors) may be required for effective suppression of SA-associated signaling pathways. Studies in tomato also failed to validate the suppression of SA signaling by COR and its components (Uppalapati et al., 2005). Nonetheless, ongoing experiments will likely provide us more information on the modulation of SA-mediated defense responses by COR and its components. The gene expression analyses by a sensitive technique like RT-qPCR provided us with information on the differences in the responses of the two *Brassica* spp. to exogenous application of COR and its components.

CHAPTER V

GENERAL CONCLUSIONS

Coronatine acts as a virulence factor of *Pst* DC3000 in edible *Brassica* spp.

Coronatine (COR) is a non-host-specific phytotoxin produced by numerous pathovars of *Pseudomonas syringae,* including *Pst* DC3000. The toxin is composed of a polyketide, coronafacic acid (CFA) linked by an amide bond to an ethylcyclopropyl amino acid, coronamic acid (CMA). The toxin acts as a virulence factor of *P. syringae* on many plants such as tomato, soybean, ryegrass and *A. thaliana* (Bender *et al.*, 1987; Budde and Ullrich, 2000; Mittal and Davis, 1995; Sato *et al.*, 1983). Although previous studies have suggested a potential role of the toxin in crucifers (*Brassica* spp.), the contribution of COR in the bacterial virulence and modulation of defense responses in these plants were not previously investigated.

The current study focuses on the exploration of the role of COR and its components in the virulence of *Pst* DC3000 on two edible species of brassicas, viz., collard (*Brassica oleracea* var. *viridis* cv. Vates) and turnip (*B. oleracea* var. *utilis* cv. Alltop). In this study, the role of COR was investigated using three biochemically-defined mutants of *Pst* DC3000: DB29 (CFA^- CMA^-); DB4G3 (CFA^- CMA^+); and AK7E2 (CFA^+ CMA^-) (Brooks *et al.*, 2004). Inoculation of collard with the COR-producing *Pst* DC3000 resulted in early and severe chlorosis, water

soaking and anthocyanin accumulation. On turnip inoculated with *Pst* DC3000, symptoms included a delayed and diffuse chlorosis and necrosis. When collard leaves were inoculated with the three bacterial mutants (DB4G3, AK7E2, and DB29), the symptoms were attenuated. Although DB29 and DB4G3 did not induce notable symptoms on turnip other than very mild chlorosis or necrosis, AK7E2 produced a considerable amount of chlorosis and necrosis on turnip, which suggests the possible production of coronafacoyl conjugates in this host. In other words, the CFA moiety might have conjugated with an amino acid in turnip plants forming other chlorosis-inducing coronafacoyl compounds (Mitchell and Ford, 1998). Collectively, these results imply that the CFA component and the intact COR toxin are required for the appearance of disease symptoms in turnip and collard, respectively.

Bacterial growth kinetics showed that *Pst* DC3000 as well as all the COR-defective mutants multiplied to similar levels in collard, implying that the toxin is not required for the persistence of the bacterium *in planta*. However, in turnip, *Pst* DC3000 maintained a higher population than the COR^- mutants throughout the sampling period, indicating a role for COR in the multiplication of the pathogen in this host.

The levels of chlorophyll in *Pst* DC3000 inoculated collard and turnip were 20 to 30%, respectively, of that of the mock-treated (water) control (Chapter III). Real-time PCR analysis revealed that the COR-producing *Pst* DC3000 induced the expression of the *CORI1* (chlorophyllase) gene in both collard (1 h) and turnip plants (12 h). Furthermore, AK7E2 (CFA^+ CMA^- mutant) also induced

CORI1 gene expression in turnip, which correlates with the ability of this mutant to cause chlorosis. *CORI1* has been characterized in *A. thaliana* and was rapidly induced in response to methyl jasmonate and COR (Benedetti *et al.*, 1998; Benedetti and Arruda, 2002; Tsuchiya *et al.*, 1999). Our observations on chlorophyll degradation support earlier studies suggesting the association of COR with the chloroplasts and the down-regulation of photosynthetic genes in COR-treated tissue (Palmer and Bender, 1995; Uppalapati *et al.*, 2005). Collectively, these results show that COR has distinct roles with respect to virulence in collard and turnip.

Coronatine modulates the major defense pathways in edible *Brassica* spp.

COR is reported to influence the salicylic acid (SA) and jasmonic acid (JA) pathways in plants (Block *et al.*, 2005; Glazebrook *et al.*, 2003; Kloek *et al.*, 2001; Kunkel and Brooks, 2002; Schmelz *et al.*, 2003; Uppalapati *et al.*, in preparation). In the current study, we investigated the effect of COR on the SA and JA pathways by monitoring the expression of *PR-1* and *LOX2*, which are markers for SA and JA, respectively (Glazebrook, 2001; Liechti and Farmer, 2002; Zhao *et al.*, 2003). *Pst* DC3000 and the single mutants (DB4G3 and AK7E2) did not induce significant expression of *PR-1* in collard. Maximal expression of *PR-1* was detected at 6 and 12 h respectively in DB29-inoculated collard and turnip, suggesting that COR suppresses SA-mediated defense (Chapter III). This finding suggests that COR may interfere with SA signaling and thus promote pathogen virulence (Kloek *et al.*, 2001). In turnip, DB4G3 (CFA^- CMA^+) induced *PR-1*

expression, thus signifying the function of CFA in suppressing SA-defense in this host. It is remarkable that maximal expression of *LOX2* occurred as early as 15 or 30 min after with *Pst* DC3000 inoculation on collard and turnip respectively.

Another observation that implicates CFA in turnip is the flux in *LOX2* gene expression observed in the AK7E2 (CFA^+ CMA^-) mutant. Since COR and CFA (in turnip) both have structural similarities with JA, they might be perceived similarly by these hosts and share a common receptor (Uppalapati *et al.*, 2005; Zhao *et al.*, 2003). The activation of JA-associated defense may be a part of the general virulence strategy of the pathogen. The antagonistic interaction between the JA and SA signaling pathways further suggests that the early activation of JA by *Pst* DC3000 might lead to the suppression of SA-associated defense and hence promote disease development.

Response of edible *Brassica* spp. to exogenous application of purified CFA, CMA and COR

In several prior investigations, the application of COR caused chlorosis and the loss of chlorophyll in different plants (Kenyon and Turner, 1992; Palmer and Bender, 1995; Uppalapati *et al.*, 2005). Collard and turnip plants treated with 0.2 nmol COR developed chlorosis at days 3 and 5, respectively (Chapter IV). There were distinctly different patterns of chlorosis on these plants that could be due to differences in the perception of COR by collard and turnip. The chlorophyll levels of COR treated collard and turnip was reduced to 30 and 50%, respectively of that in healthy leaves. The expression of the *CORI1* gene earlier

in collard (6 h) than in turnip (12-24 h), as shown by real-time PCR analysis is consistent with the differential timing of chlorosis in these hosts. The induction of *CORI1* by COR was reported formerly (Benedetti and Arruda, 2002; Brooks *et al.*, in press; Tsuchiya *et al.*, 1999). The application of equimolar concentrations of CFA and CMA did not induce visible changes on either plant or a significant reduction in chlorophyll content. There was no obvious difference in the expression of *CORI1* in CFA or CMA treated collard or turnip as compared to the water control. Thus, the intact toxin (when applied as a purified compound) is required for chlorosis on both these *Brassica* spp. In experiments using bacterial mutants (Chapter III), turnip plants inoculated AK7E2 (*cmaA* mutant; CFA^+ CMA^-) developed chlorosis and contained reduced chlorophyll levels (Chapter III). These observations were attributed to the potential ability of CFA to form coronafacoyl conjugates with amino acids in turnip. However, since chlorosis was not observed when exogenous CFA was applied to turnip, the presence of the bacterium may be required to elicit chlorosis. For example, the bacterium may provide the coronafacoyl ligase enzyme for conjugating CFA with other amino acids, and the absence of this enzyme may be responsible for the lack of chlorosis when pure CFA was applied to the plants.

As explained in the bacterial inoculation experiments, the exogenous application of COR induced the expression of *LOX2* in both collard and turnip within 30 min after treatment. This could be explained by the similarity of COR to JA and associated molecules (Block *et al.*, 2005; Feys *et al.*, 1994). The *LOX2* induction by CFA and CMA treated collard plants was very similar to the mock

(water) treated control. But in turnip leaves, *LOX2* was expressed within 1 h after treatment with CFA, suggesting a potential role for CFA in modulating JA-mediated defenses in turnip. Microarray analysis in tomato revealed that the CFA moiety regulated 40% of the COR-modulated genes and most of the JA-responsive genes (Uppalapati *et al.*, 2005). However, *LOX2* induction in turnip by CMA was not significantly different from that in mock-treated water control. Nonetheless, the expression of *PR-1*, a marker used to follow SA signaling, was not induced by any of these treatments. Possible explanations include suppression of the SA pathway by COR and its components, or the failure of the compounds to influence the pathway when applied in purified form. Detailed investigations are underway to monitor the influence of COR and its components on *PR-1* gene expression after pre-inducing the gene by exogenous salicylic acid.

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APPENDIX

Effect of Coronatine, Coronafacic acid and Coronamic acid

on Brassica seedlings

SUMMARY

Coronatine (COR), when applied as a purified compound, inhibited the growth of collard and turnip seedlings. Lower concentrations of COR inhibited the growth of collard seedlings, while relatively higher concentrations of COR reduced seedling size and dry weight in turnip. The components of the toxin, coronafacic acid (CFA) and coronamic acid (CMA) did not induce any changes on the seedlings of either *Brassica* spp.

INTRODUCTION

Coronatine (COR) is a chlorosis-inducing phytotoxin produced by several *Pseudomonas syringae* pathovars and has diverse effects on plants. The inhibition of root growth is one of the many biological effects of COR (Sakai, 1980; Feys *et al*., 1994). Additionally, COR induces anthocyanin accumulation in *Arabidopsis thaliana* and tomato seedlings (Feys *et al*., 1994; Uppalapati *et al*., 2005.) In tomato, root inhibition was observed in a dose-dependent manner. In this study, we attempt to investigate whether the seedlings of two *Brassica* spp. (collard and turnip) responded differently to COR, CFA, and CMA. The

results show that COR, but not its components, induce root inhibition on *Brassica* spp. at the concentrations used. The phenotypic response, root length and dry weight measurement reveal that the growth of collard seedlings was inhibited at a lower concentration of COR (0.2 nmol) and turnip seedling growth was dramatically reduced at higher concentrations (1 nmol).

EXPERIMENTAL PROCEDURES

Seeds of collard (*B. oleraceae* var. *viridis* L. cv. Vates) and turnip (*B. rapa* var. *utilis* (DC) Metzg. cv. Alltop) were germinated on water-saturated filter paper placed in Petri plates for 3 days at room temperature. The seedlings were then treated with two different quantities (0.2 nmol and 1 nmol) of CFA, CMA and COR. The treatments were made by diluting the required amount of the individual compounds in 1 ml of sterile distilled water. Mock inoculations were made by applying 1 ml of sterile distilled water. The seedlings were then incubated for 4 days at room temperature. The seedlings were then photographed, and seedling lengths were measured. The seedlings were then placed on dry paper towel in Petri plates and kept at 37^0C. A reduction in the moisture content was observed starting at 2 days. The dry weight was taken daily until a constant value (for about a week) was obtained. The average values for all parameters were calculated for four seedlings each. The experiments were repeated twice with similar results.

RESULTS

The application of 0.2 nmol and 1 nmol COR inhibited root growth in collard seedlings, while the inoculation of 0.2 or 1 nmol CFA and CMA did not induce any visible reduction in seedling growth (Fig.24 A, B). COR treatment (0.2 nmol) reduced collard seedling length to 2.4 cm when compared to the mock-inoculated seedlings, which were 6.7 cm. There was also a considerable decline in the root growth after application of 1 nmol COR (2 cm seedling length) as compared to the control (6.6 cm) (Fig. 25A). The dry weight of the COR-inoculated (0.2 nmol) collard seedlings was substantially lower (1.8 mg) as compared to the mock-inoculated control (4.3 mg). When collard seedlings were inoculated with 1 nmol COR, dry weight was 1.5 mg as compared to 4.3 mg for control seedlings (Fig. 25B). Nevertheless, the difference in the reduction in seedling length and dry weight of collard seedlings treated with the two concentrations of COR was not very different.

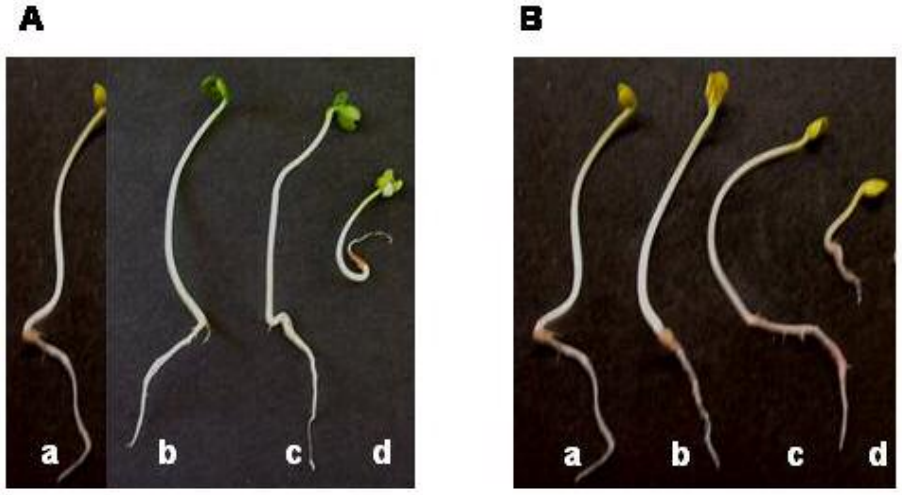


Fig.24. Seedlings of collard treated with **(A)** 0.2 nmol **and (B)** 1 nmol of: (a) water; (b) CFA; (c) CMA; and (d) COR. The compounds were applied on seedlings grown on water-saturated filter paper for 3 days at 24^0C. The seedlings were further maintained in the same conditions for another 3 to 4 days. Photographs were taken 4 days after treatment.

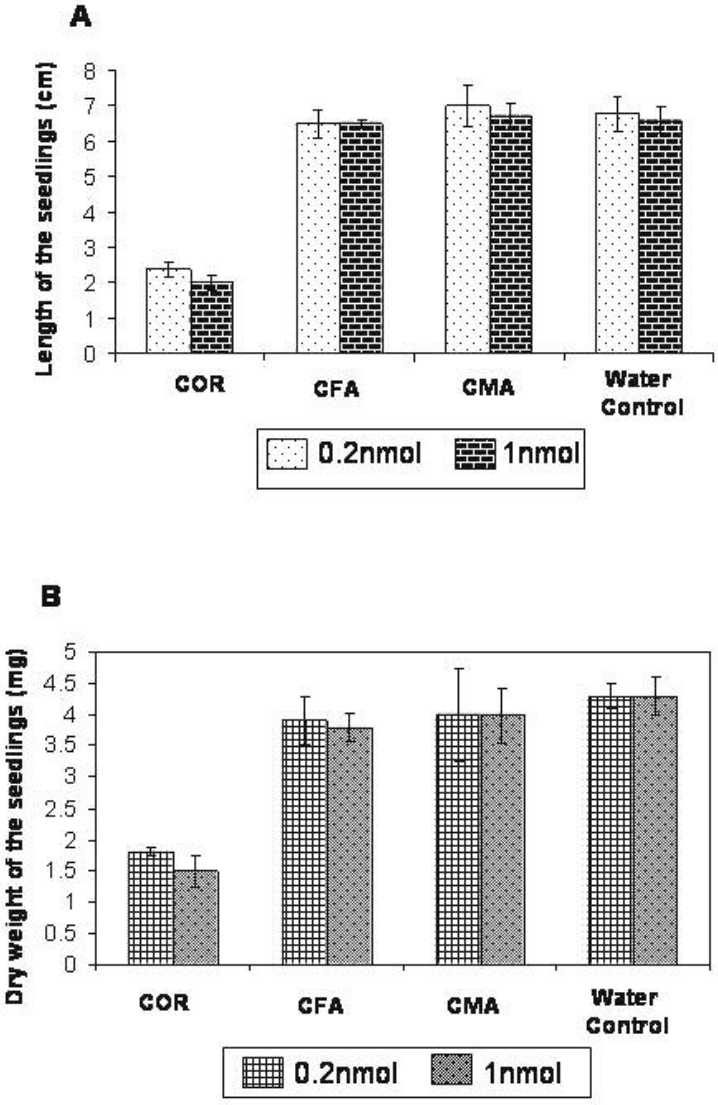
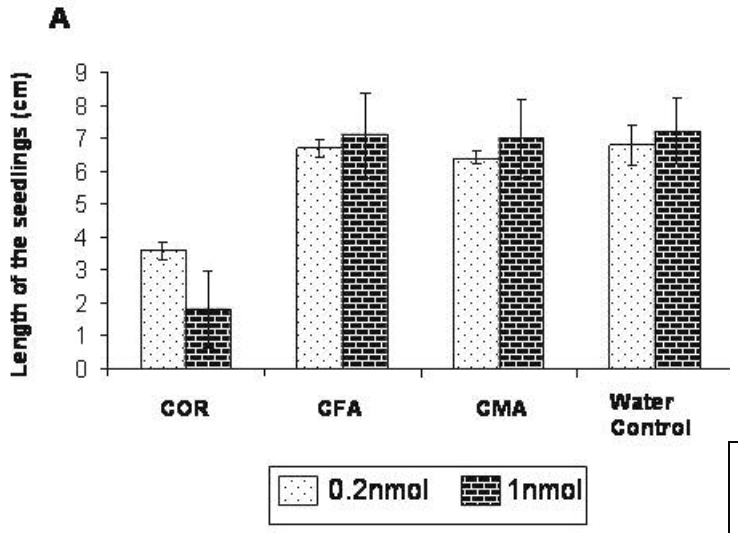


Fig.25. (**A**) Length and (**B**) dry weight of collard seedlings treated with 0.2 nmol or 1 nmol COR, CFA, CMA and water control. The experiment was repeated twice with similar results.

In turnip plants, treatment with 0.2 nmol COR reduced root growth (Fig. 26A). The length of COR-treated turnip seedling was 3.6 cm, which was shorter than the water-treated control seedlings (6.5 cm) (Fig. 27A). However, application of 1 nmol COR on turnip seedlings resulted in very tiny seedlings (Fig. 26B). The length of the seedlings was reduced to 1.8 cm, while the mock-inoculated seedlings were 7.2 cm long. There was a significant difference in the extent of seedling growth in turnip treated with the higher (1 nmol) concentration

of the toxin. There was also reduction in dry weight after COR treatment (0.2 nmol) on turnip (2.3 mg) as compared to treatment with water (dry weight of 3.4 mg). The seedling dry weight was 1.3 mg after treatment with 1 nmol COR as compared to a higher weight for the water control (3.6 mg) (Fig. 27B). The root growth, length and dry weight were considerably lower than that of the turnip seedlings treated with 0.2 nmol COR. The application of either concentration (0.2 and 1 nmol) of CFA or CMA failed to induce any phenotypic effects or differences in seedling growth and dry weight and were similar to the mock treatment.

Fig.26. Seedlings of turnip treated with **(A)** 0.2 nmol **(B)** 1 nmol of: (a) water; (b) CFA; (c) CMA; and (d) COR. The compounds were applied on seedlings grown on water-saturated filter paper for 3 days at 24^0C, and the seedlings were further maintained at the same conditions for another 3 to 4 days. Photographs were taken 4 days after treatment.



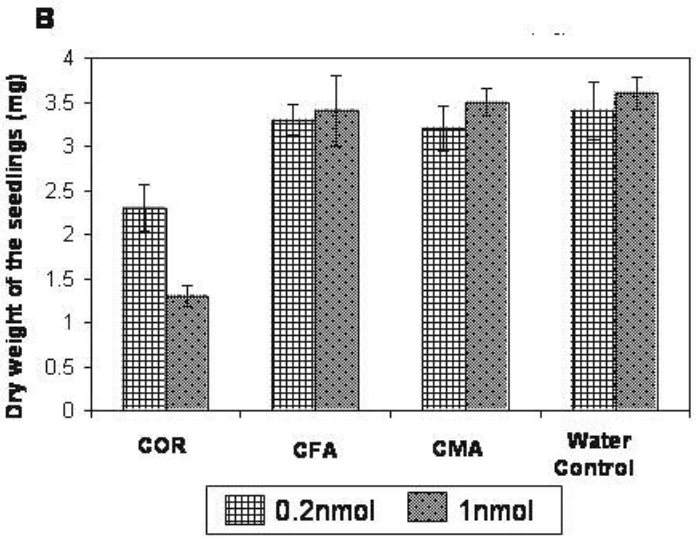


Fig.27. (**A**) Length and (**B**) dry weight of turnip seedlings treated with 0.2 nmol and 1 nmol of COR, CFA, CMA and water control. The experiment was repeated twice with similar results.

DISCUSSION

The seedling assays indicate that the whole toxin (COR), but not its components are capable of inducing obvious changes on both collard and turnip seedlings. The phenotypic response observed with COR treatment was a reduction in growth and dry weight of seedlings. CFA and CMA did not impact seedling growth, which correlates with the lack of symptom development in the leaves of both *Brassica* spp. by these two components (Chapter IV). In previous studies, CMA did not inhibit tomato seedling growth, but CFA inhibited tomato root length when applied at higher concentrations (e.g. 20 nmol) (Uppalapati *et al*., 2005). In the present study, we find that COR is more biologically active than CFA and CMA in both collard and turnip seedlings. Also, it is important to note that there was no appearance of anthocyanin in any of the treatments, unlike results observed with *A. thaliana* and tomato (Feys *et al*., 1994; Uppalapati *et al*., 2005.) The appearance of anthocyanins in response to COR may be host and/or tissue-specific (Feys *et al*., 1994).

In this study, the effect of two different concentrations of purified COR, CFA and CMA were examined on collard and turnip seedlings. Application of 0.2 nmol of COR was sufficient to reduce the length and dry weight of collard seedlings by 2.8 and 2.4-fold respectively, when compared to the water-treated control. Although 0.2 nmol COR inhibited root length in turnip seedlings, the seedling length and dry weight were decreased by only 1.8- and 1.4-fold relative to the water-treated control. Another interesting observation is the variation in

observed effects according to the concentration of toxin applied. When 1 nmol of COR was applied, collard seedlings showed a 3.3- and 2.8-fold decrease in seedling length and dry weight, respectively, in comparison to the water-treated control. Interestingly, in turnip, the application of 1 nmol COR induced an even higher reduction in the length and dry weight. Turnip seedling length and dry weight were reduced by 4 and 2.8-fold, respectively, as compared to the control. Hence, in collard, a lower concentration of the toxin is enough to cause significant root inhibition, while a higher concentration of COR induces inhibition of turnip seedling growth. Altogether, these results indicate that there are notable differences in the effect of COR on seedlings of collard and turnip.

VITA

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Candidate for the Degree of

Doctor of Philosophy

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Major Field: Plant Pathology

Scope and Method of Study: The phytotoxin coronatine (COR), produced by different strains of *Pseudomonas syringae* has two moieties: coronamic acid (CMA) and coronafacic acid (CFA). The bacterium *P. syringae* pv. *tomato* DC3000 is pathogenic on tomato, Arabidopsis and edible species of brassicas. This study is focused on understanding the roles of COR and its components in the bacterial virulence on two major cultivated brassicas, collard and turnip. Furthermore, defense gene expression in these two plants in response to COR-defective biosynthetic mutants as well as the purified toxin and its components were investigated.

Findings and Conclusions: COR contributed to the virulence of *Pst* DC3000 in both collard and turnip by contributing to the symptoms in both plants and bacterial multiplication in turnip. Gene expression studies showed that COR stimulated the activation of *CORI1* and *LOX2* genes and suppression of *PR-1* gene in both plants. The treatment of turnip with AK7E2 (CFA^+ CMA^-) resulted in chlorosis, expression of *CORI1* and *LOX2* genes and *PR-1* suppression; suggesting the role of CFA in defense modulation in this plant. The exogenous application of purified COR also induced chlorophyll degradation and JA activation on both plants, whereas the CFA component induced JA-mediated defense in turnip. The activation of the jasmonic acid (JA) pathway by COR may be a part of the general virulence strategy of the pathogen. The results of this study suggest that CFA component has some role in the interaction of *Pst* DC3000 with turnip but not collard.

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