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Mini review

Chlorosis-inducing phytotoxins produced by *Pseudomonas syringae*

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Abstract

Pseudomonas syringae causes a wide range of symptoms on plants including blights, leaf spots, and galls. Phytotoxins generally enhance the virulence of phytopathogenic *P. syringae*, and their synthesis can substantially increase disease severity. Although several *P. syringae* phytotoxins cause chlorosis (coronatine, phaseolotoxin, and tabtoxin), they are synthesized by unrelated biosynthetic pathways and have completely different modes of action. Phaseolotoxin and tabtoxin inhibit ornithine carbamoyltransferase and glutamine synthetase, respectively, whereas coronatine functions as a mimic of methyl jasmonate in some plant species. This review focusses on the mode of action, genetics, biosynthesis and regulation of coronatine, tabtoxin, and phaseolotoxin. Current techniques used to detect these toxins and phytotoxin-producing *P. syringae* pathovars are discussed. The utilization of toxin resistance genes in the development of transgenic plants with phytotoxin tolerance is also reviewed.

Abbreviations: CFA – coronafacic acid; CFA-ile – coronafacoylisoleucine; CFA-val – coronafacoylvaline; CMA – coronamic acid; COR – coronatine; OCTase – ornithine carbamoyltransferase; PSorn – octicidine; TβL – tabtoxinine-β-lactam.

Introduction

Phytotoxins may be host-specific and exhibit the same specificity as the producing pathogen or nonhost-specific with a wider host range of activity than the producing pathogen. In general, the phytotoxins produced by *P. syringae* are nonhost-specific and cause symptoms on many plants which cannot be infected by the toxin-producing pathogen. The chlorosis-inducing phytotoxins discussed in this review (coronatine, phaseolotoxin, and tabtoxin) are not required for pathogenicity in *P. syringae*; instead they function as virulence factors, and their production results in increased disease severity. This review will focus on the genetics, mode of action, and detection of coronatine, phaseolotoxin, and tabtoxin.

Coronatine

Coronatine (COR) consists of two distinct structural components: (1) the polyketide coronafacic acid (CFA) and (2) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara et al., 1977; Mitchell 1985a; Parry et al., 1994). In general, coronatine is the predominant coronafacoyl compound synthesized by COR producers and also the most toxic; however, other coronafacoyl compounds may be synthesized which contain various amino acid substituents conjugated to CFA via an amide linkage (Mitchell, 1985b; Mitchell and Young, 1985). Our current understanding of the COR biosynthetic pathway is summarized in Figure 1.

Biological effects of coronatine and mode of action

The primary symptom elicited by COR is a diffuse chlorosis which can be induced on a wide variety of plant species. COR also induces hypertrophy, inhibits root elongation, and stimulates ethylene production in some but not all plant species (Kenyon and Turner, 1992; Sakai et al., 1979). Several researchers have investigated the structural and functional homologies between COR and methyl jasmonate (MeJA), a plant

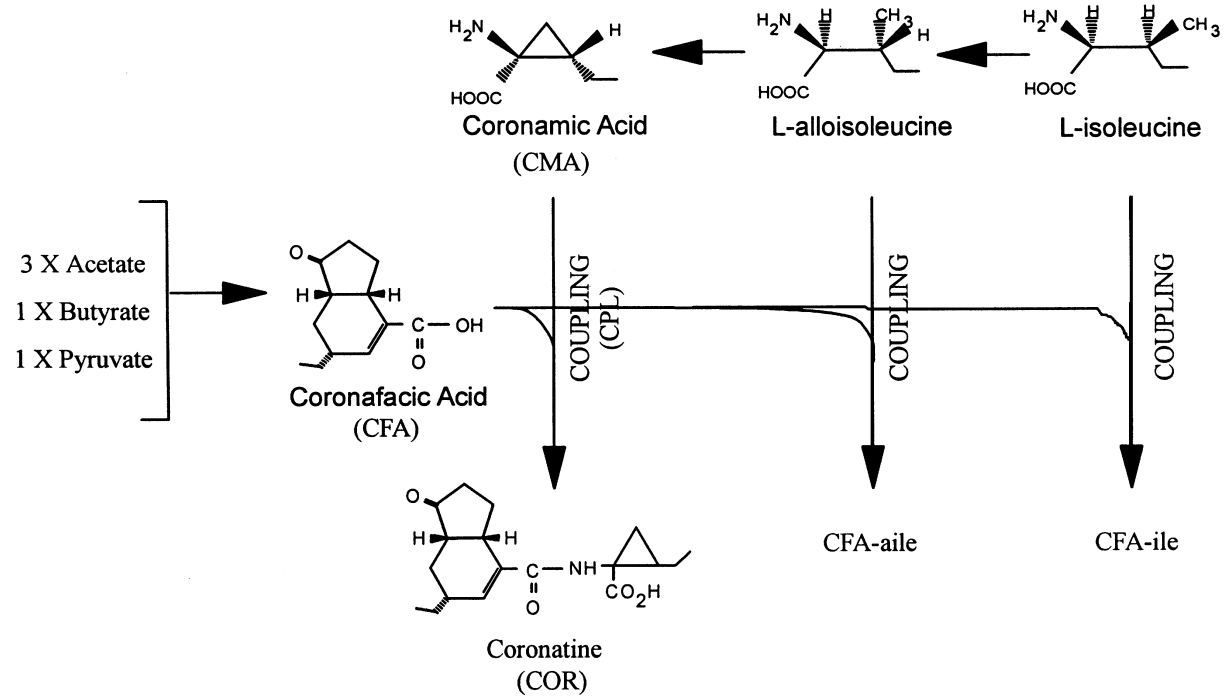

Figure 1. Biochemical pathways involved in the synthesis of coronatine (COR) and coronafacoyl compounds in *P. syringae* pv. *glycinea* PG4180. COR consists of a polyketide component, coronafacic acid (CFA), coupled (CPL) via amide bond formation to an amino acid component, coronamic acid (CMA). CFA is synthesized as a branched polyketide from three acetate units, one pyruvate, and one butyrate unit via an unknown sequence of events (Parry et al., 1994). CMA is derived from isoleucine via alloisoleucine and cyclized by an unknown mechanism (Parry et al., 1994). CMA functions as an intermediate in the COR biosynthetic pathway, which indicates that cyclization of L-alloisoleucine to form CMA occurs before CFA and CMA are coupled (Mitchell et al., 1994). The coronafacoyl analogues, CFA-ile and coronafacoylalloisoleucine (CFA-aile), result from amide bond formation between CFA and isoleucine and alloisoleucine, respectively, and are not further utilized in the synthesis of COR.

growth regulator derived from the octadecanoid signaling pathway (Wasternack and Parthier, 1997). COR and MeJA induce analogous biological responses in *Arabidopsis* seedlings, *Eschscholtzia californica* cell cultures, and potato tissue; these results have led researchers to suggest that COR functions as a molecular mimic of the octadecanoid signaling molecules produced by higher plants (Feys et al., 1994; Koda et al., 1996; Weiler et al., 1994). Palmer and Bender (1995) used light microscopy to compare the effects of COR, CFA, and MeJA on tomato tissue. Although all three compounds induced the production of proteinase inhibitors, only COR caused cell wall thickening, changes in chloroplast structure, and chlorosis; CFA and MeJA did not induce these changes in tomato tissue (Palmer and Bender, 1995). Consequently, the CMA moiety, or perhaps the amide linkage between CFA and CMA, may impart additional biological activities to COR in tomato. Therefore, COR does not function solely as a molecular mimic of MeJA in tomato, and the mechanism of action for COR may remain unclear until putative receptors for the toxin are localized in various plant species.

Genetic studies

Production of the phytotoxin COR has been demonstrated in *P. syringae* pvs. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato* which infect ryegrass, soybean, crucifers, *Prunus* spp., and tomato, respectively (Mitchell, 1982; Mitchell et al., 1983; Wiebe and Campbell, 1993). Tn*5* mutagenesis has been used to obtain coronatine-defective (COR^-) mutants of pvs. *atropurpurea*, *glycinea*, *morsprunorum*, and *tomato*, and COR was shown to have a distinct role in virulence (Bender et al., 1987; Bender et al., 1991; Mittal and Davis, 1995; Sato et al., 1983). Although COR^- mutants were still pathogenic, they produced

smaller necrotic lesions and failed to achieve the population levels attained by COR-producing strains. These results indicate that COR synthesis contributes significantly to lesion expansion and multiplication of the bacterium in the plant (Bender et al., 1987; Feys et al., 1994; Mittal and Davis, 1995). Although the COR gene cluster has been frequently associated with large (80–110 kb) plasmids, these genes can also be chromosomally encoded (Bender et al., 1991; Cuppels and Ainsworth, 1995; Liang et al., 1994; Sato et al., 1983).

COR biosynthesis has been intensively studied in *P. syringae* pv. *glycinea* PG4180. Tn*5* mutagenesis and plasmid transformation experiments indicated that the COR biosynthesis genes in PG4180 are encoded by a 90-kb plasmid designated p4180A (Bender et al., 1991; 1993). The 32-kb COR gene cluster contains two distinct regions which encode the structural genes for CMA and CFA biosynthesis, and these are separated by a 3.4-kb regulatory region (Bender et al., 1996). Nucleotide sequence analysis indicated that the CMA region contained four genes designated *cmaA*, *cmaB*, *cmaT*, and *cmaU*, and further analyses of the predicted protein products indicated that CMA is produced via a thiotemplate mechanism by a modified peptide synthetase (Budde et al., 1998; Ullrich and Bender, 1994).

Complementation experiments utilizing CFA$^-$ mutants and an extensive series of subclones demonstrated that the CFA region was organized as a single 19-kb transcriptional unit (Liyanage et al., 1995a). Nucleotide sequencing revealed the presence of 11 ORFs within the CFA transcript, including *cfl*, *cfa1-cfa9*, and *tnp1* (Liyanage et al., 1995b; Penfold et al., 1996; Rangaswamy et al., 1998). The translational products of *cfl* and *cfa5* showed relatedness to enzymes which activate carboxylic acids by adenylation (acyl CoA ligases); however, their role in CFA biosynthesis remains unclear (Liyanage et al., 1995b; Penfold et al., 1996; Rangaswamy et al., 1997). The translational products of *cfa1*, *cfa2*, and *cfa3* showed relatedness to acyl carrier protein, fatty acid dehydratase, and β-ketoacyl synthetase, respectively, indicating that these genes are involved in assembly and modification of the polyketide, CFA (Penfold et al., 1996). The continued sequencing of the CFA region revealed the presence of two large ORFs (*cfa6* and *cfa7*) which exhibit a high degree of similarity to 6-DEB synthase, the enzyme responsible for the synthesis of 6-deoxyerythronolide B (an intermediate in the pathway to the polyketide erythromycin) (C Bender, unpubl). The predicted translational product of *cfa8* showed similarity to crotonyl CoA reductases from *Streptomyces* spp., and *cfa9* showed similarity to the thioesterases involved in the synthesis of gramicidin, tyrocidine, and tylosin (Rangaswamy et al., 1998). Although the sequence of events involved in CFA assembly remain unclear, our results now indicate that CFA is produced by a unique combination of mono- and multifunctional enzymes, indicating a cooperative activity which is unusual in polyketide synthesis.

Regulation of coronatine production

COR biosynthesis in *P. syringae* pv. *glycinea* PG4180 is temperature-sensitive, with maximal production at 18 °C and negligible yields at 30 °C (Palmer and Bender, 1993). This response to temperature is consistent with symptom development in the field, since *P. syringae* pv. *glycinea* is predominantly a cool-weather pathogen. CFA and CMA were also subject to the same pattern of temperature control, with optimal production at 18 °C (Ullrich and Bender, 1994; Palmer 1995). Recently, Rohde et al. (1998) showed that COR production was thermoregulated in pvs. *atropurpurea*, *maculicola*, *morsprunorum*, and *tomato*, suggesting that temperature is a common regulatory control for COR biosynthesis in other pathovars of *P. syringae*.

The production of both CFA and CMA in PG4180 is regulated at the transcriptional level by temperature. Transcriptional fusions of the CFA and CMA promoter regions to a promoterless glucuronidase gene indicated that transcriptional activity in both gene clusters was maximal at 18 °C and significantly less at 28 °C (Rangaswamy et al., 1997; Ullrich and Bender, 1994). The higher level of transcriptional activity for the CMA and CFA biosynthetic promoters at 18 °C helps explain why COR production is optimal at this temperature.

A regulatory region was isolated which controls both CFA and CMA production; the nucleotide sequence of this region revealed the presence of three genes, *corP*, *corS*, and *corR* (Ullrich et al., 1995). The deduced amino acid sequence of *corP* and *corR* indicated relatedness to response regulators which function as members of two-component regulatory systems, and the translational product of *corS* showed similarity to histidine protein kinases which function as environmental sensors (Ullrich et al., 1995). Complementation analysis using a *corR* mutant, PG4180.P2, and transcriptional fusions to a promoterless glucuronidase gene (*uidA*) indicated that CorR functions as a positive

regulator of COR gene expression (Peñaloza-Vázquez and Bender, 1998).

Several approaches have been utilized to investigate the potential stimulation of COR synthesis by host plants. Palmer and Bender (1993) amended the growth medium for PG4180 with extracts from soybean tissue or with plant-derived secondary metabolites but found no evidence that these substances substantially increased COR production *in vitro*. In a subsequent study, the activity of *cmaA*::*uidA* and *cfl*::*uidA* transcriptional fusions was compared *in vitro* and in soybean leaves; however, there was no evidence that COR gene expression in PG4180 was higher in plant tissue (C Bender, unpubl). In contrast, Ma et al. (1991) showed that COR biosynthesis in *P. syringae* pv. *tomato* DC3000 is plant-inducible, suggesting that the signals for induction of COR synthesis differ in PG4180 and DC3000. Gene fusions indicated that a single transcriptional unit in DC3000 designated CorII was expressed at a higher level *in planta* than *in vitro*. More recent results indicate that shikimic and quinic acids may be signals for COR gene induction in DC3000 (Li et al., 1998).

Tabtoxin

P. syringae pvs. *tabaci*, *coronafaciens*, and *garcae* produce tabtoxin, a monocyclic β-lactam (Mitchell, 1991). The dipeptide toxin contains tabtoxinine-β-lactam (TβL) linked by a peptide bond to threonine (Figure 2). Although tabtoxin is the primary metabolite produced, the chlorosis-inducing activity occurs only after hydrolysis of the peptide bond by aminopeptidase activity, a reaction which releases TβL, the toxic moiety (Durbin and Uchytil, 1984; Levi and Durbin, 1986).

Mode of action for tabtoxin

TβL has been shown to irreversibly inhibit glutamine synthetase (Thomas et al., 1983), an enzyme which is required for the efficient detoxification of ammonia. Many deleterious effects have been attributed to ammonia accumulation in plants, including disruption of the thylakoid membrane of the chloroplast and uncoupling of photophosphorylation (Turner and Debbage, 1982). Protection of the bacteria from the toxin has been associated with the adenylation of glutamine synthetase, which renders the target enzyme less susceptible to inactivation by TβL (Knight et al., 1986). Another

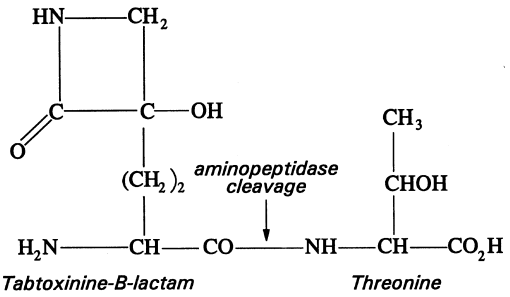

Figure 2. Structure of tabtoxin which consists of the toxic moiety, tabtoxinine-β-lactam (TβL), linked via an amide bond to threonine. The arrow shows the site of aminopeptidase cleavage which releases TβL.

potential detoxification mechanism involves the synthesis of β-lactamases which hydrolyze the β-lactam ring of TβL to liberate the nontoxic metabolite, tabtoxinine (Knight et al., 1987).

Genetics of tabtoxin production

Although TβL is associated with the symptoms of wildfire disease on tobacco and halo blight of oats, it is generally considered to be a virulence factor rather than an essential component of these diseases. For example, *P. syringae* pv. *angulata*, which induces necrotic spots on tobacco leaves without producing chlorotic halos, is considered to be a spontaneous Tox$^-$ derivative of *P. syringae* pv. *tabaci* (Kinscherf et al., 1991). Similarly, *P. syringae* pv. *striafaciens* is thought to be a nontoxigenic derivative of *P. syringae* pv. *coronafaciens*, causal agent of halo blight of oats (Willis et al., 1991). The natural occurrence of tabtoxin-defective strains is caused by the instability of the tabtoxin gene cluster which excises from the chromosome at fairly high frequencies (Kinscherf et al., 1991). Kinscherf and coworkers (1991) cloned a 31-kb DNA fragment which conferred tabtoxin production to *P. syringae* strains BR2 and Cit7, suggesting that all genes necessary for tabtoxin synthesis in *P. syringae* were clustered in this 31-kb region.

Unlike coronatine and phaseolotoxin, the loss of tabtoxin production has varied effects on the pathogenicity of *P. syringae*. Tabtoxin-deficient mutants of *P. syringae* pv. *tabaci* or *P. syringae* pv. *coronafaciens* failed to induce chlorosis, but retained the ability to induce necrotic lesions (Willis et al., 1991). However, tabtoxin-defective mutants of the bean pathogen, *P. syringae* BR2, produced no detectable symptoms on bean plants (Kinscherf et al., 1991).

Biosynthesis and regulation of tabtoxin

The incorporation of ^{13}C-labeled compounds demonstrated that the biosynthetic precursors for tabtoxin included L-threonine and L-aspartate for the side chain and pyruvic acid and the methyl group of L-methionine for the β-lactam moiety (Roth et al., 1990; Unkefer et al., 1987). A biosynthetic model for the formation of TβL resembles that of lysine, where the first dedicated step is the DapA-catalyzed condensation of aspartic acid semialdehyde with pyruvate to form L-2,3, dihydropicolinate (DHDPA). The following step is catalyzed by DapB and involves the reduction of DHDPA to L-2,3,4,5-tetrahydropicolinate (THDPA).

The discovery of *tabA* provided the first genetic evidence supporting the origin of tabtoxin from the lysine biosynthetic pathway (Engst and Shaw, 1992). The translational product of *tabA* showed relatedness to *lysA* which encodes diaminopimelate decarboxylase in bacteria. Although *tabA* was not required for lysine biosynthesis, Engst and Shaw (1992) proposed that the *tabA* translational product is an enzyme which recognizes a substrate analogue of a compound in the biosynthetic route to lysine. Recently, Liu and Shaw (1997a) demonstrated that *dapB* was required for both DAP and TβL synthesis. Their data indicate that the lysine and TβL pathways most likely diverge after THDPA synthesis and before DAP formation, which agrees with the hypotheses of other researchers (Roth et al., 1990; Unkefer et al., 1987).

The deduced product of *tabB*, also located in the TβL biosynthetic region (Liu and Shaw, 1997b), showed relatedness to *dapD*, a gene encoding L-2,3,4,5-tetrahydrodipicolinate succinyl coenzyme A succinyltransferase (THDPA-ST). Complementation studies and enzymatic assays indicated that *tabB* encodes a product with THDPA-ST activity. TabB is presumed to be an acetyltransferase that converts an unknown compound to an acetyl derivative which is further metabolized to TβL (Liu and Shaw, 1997b). This is consistent with an earlier proposal which suggested that acetylated intermediates are involved in tabtoxin biosynthesis (Feistner et al., 1991).

In summary, DapB is essential for both lysine and tabtoxin biosynthesis, and THDPA may be an intermediate in both pathways. Three genes have been characterized in the 31-kb region which contains all genes necessary for TβL synthesis and tabtoxin resistance: *tabA*, *tabB*, and a third gene, *tblA* (Engst and Shaw, 1992; Liu and Shaw, 1997b; Barta et al., 1993). Although there is no obvious relationship between TblA and known polypeptides, TabA has relatedness to LysA from *E. coli* and *P. aeruginosa*, whereas TabB shows similarity to DapD.

Some progress has been made on elucidating factors which regulate tabtoxin biosynthesis in *P. syringae*. Production of TβL by *P. syringae* pv. *tabaci* was shown to require zinc, a co-factor needed for the aminopeptidase which hydrolyzes tabtoxin to release TβL (Durbin and Uchytil, 1985; Levi and Durbin, 1986). In a subsequent study, Barta et al. (1992) showed that *tblA* is regulated by the *lemA* (*le*sion *m*anifestation) gene in *P. syringae* pv. *coronafaciens*. The *lemA* locus was conserved among *P. syringae* pathovars and contained domains that are characteristic of histidine protein kinases which function as environmental sensors (Hrabak and Willis, 1992; Rich et al., 1992).

Phaseolotoxin

Phaseolotoxin is a chlorosis-inducing phytotoxin produced by *P. syringae* pvs. *phaseolicola* and *actinidiae*, which cause halo blight on legumes and bacterial canker on kiwifruit, respectively (Mitchell, 1976; Sawada et al., 1997). The structure of phaseolotoxin was initially elucidated by Mitchell (1976) with minor revisions by Moore et al. (1984), and consists of a sulphodiaminophosphinyl moiety linked to a tripeptide consisting of ornithine, alanine, and homoarginine (Figure 3A).

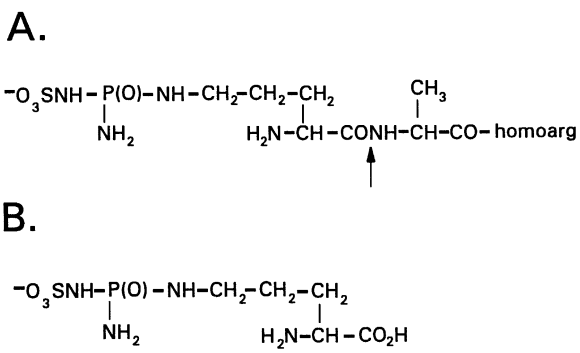

Figure 3. Structure of phaseolotoxin (**A**) and octicidine (**B**). Plant peptidases cleave phaseolotoxin (see arrow) to release the alanine and homoarginine residues, a reaction which results in octicidine formation.

Mechanism of action for phaseolotoxin

Phaseolotoxin competitively inhibits ornithine carbamoyltransferase (OCTase), which converts ornithine and carbamoyl phosphate to citrulline. Although phaseolotoxin is a reversible inhibitor of OCTase, it is hydrolyzed *in planta* by peptidases to produce N^{δ}-(N'sulpho-diaminophosphinyl)-L-ornithine, also called octicidine or PSorn (Figure 3B). Unlike phaseolotoxin, octicidine is an irreversible inhibitor of OCTase and the predominant form of the toxin in infected tissues (Mitchell and Bieleski, 1977). Inhibition of OCTase causes an accumulation of ornithine and a deficiency in intracellular pools of arginine, leading to chlorosis. *P. syringae* pv. *phaseolicola* deals with the toxic effect of phaseolotoxin by producing two isozymes of OCTase; ROCTase is resistant to the toxin, and SOCTase is sensitive. During conditions favorable to phaseolotoxin production, *P. syringae* pv. *phaseolicola* synthesizes the ROCTase isoform (Peet and Panopoulos, 1987).

Genetic studies of phaseolotoxin

The importance of phaseolotoxin in the virulence of *P. syringae* pv. *phaseolicola* was first demonstrated by Patil and co-workers (1974) who showed that Tox^{-} mutants did not move systemically in bean plants. Peet et al. (1986) reported the first cloning of genes required for phaseolotoxin biosynthesis on a cosmid clone designated pRCP17. A 2.6-kb fragment from this clone was subsequently used as a probe for detection of strains which produce phaseolotoxin (Schaad et al., 1989). pRCP17 was also shown to contain the *argK* gene which encodes ROCTase (Peet and Panopoulos, 1987).

Independently, Zhang et al. (1993) isolated a clone containing a 25-kb insert (pHK120) which restored phaseolotoxin production to Tox^{-} mutants. Complementation analysis indicated that pHK120 contained eight transcriptional units designated *phtA* through *phtH* (Zhang et al., 1993). Six ORFS were subsequently identified in the *phtE* transcript, and three of these showed significant relatedness with genes or motifs deposited in various databases (Zhang and Patil, 1997). The translational product of ORF3 was similar to acetylornithine aminotransferase (ACOAT) and ornithine aminotransferase (OAT). ACOAT catalyzes the reversible conversion of *N*-acetylglutamate-5-semialdehyde and glutamate to N^{2}-acetylornithine and 2-oxoglutarate; whereas OAT catalyzes transfer of the acetyl group of N^{2}-acetylornithine onto glutamate to yield ornithine and *N*-acetylglutamate (Cunin et al., 1986). These results suggest that ORF3 is involved in the production of ornithine, a component of phaseolotoxin (Zhang and Patil, 1997). ORF5 contained a helix-turn-helix domain and a putative leucine zipper, suggesting a regulatory role for ORF5 in phaseolotoxin production. The translational product of ORF6 showed similarity to fatty acid desaturases which generate unsaturated fatty acids for the synthesis of phospholipids (Wada et al., 1990). Independently, Hatziloukas and co-workers (1995) sequenced the 2.6-kb fragment used for detection of *P. syringae* pv. *phaseolicola* and showed that it contained a gene (designated *ptx*) related to fatty acid desaturases. Although several differences were noted between the nucleotide sequence of ORF6 and *ptx*, they appear to be the same gene. Hatziloukas and colleagues (1995) speculated that the fatty acid desaturase encoded by *ptx* may facilitate the export of phaseolotoxin across the bacterial membrane at the low temperatures conducive to phaseolotoxin production.

Biosynthesis and regulation of phaseolotoxin

Biosynthetic precursors for the N^{δ}-(N'sulpho-diaminophosphinyl) moiety of phaseolotoxin have not been identified. Märkisch and Reuter (1990) demonstrated that the homoarginine and ornithine residues of phaseolotoxin are synthesized by a transamidination reaction from arginine and lysine. The amidinotransferase had a M_{r} of about 200,000 and showed high substrate specificities for arginine and lysine in phaseolotoxin-producing strains of *P. syringae* pv. *phaseolicola*. Zhang and Patil (1997) suggested that the ORF3 product of *phtE* may catalyze the formation of the ornithine needed for phaseolotoxin production, but biochemical evidence for this function is lacking.

Temperature is a factor which regulates phaseolotoxin production in *P. syringae* pv. *phaseolicola*. Goss (1940) showed that the chlorosis associated with *P. syringae* pv. *phaseolicola* infection of bean was induced at cooler temperatures (18–20 °C) and absent at warmer temperatures (28–32 °C). Subsequent studies showed that phaseolotoxin production decreased progressively at temperatures above 18 °C (Mitchell, 1978). Rowley et al. (1993) observed the production of a repressor when *P. syringae* pv. *phaseolicola* was grown at 28 °C, a temperature unfavorable for phaseolotoxin synthesis. Production of this repressor at the

nonpermissive temperature may explain the thermoregulation of phaseolotoxin biosynthesis.

Detection of phytotoxins and toxin-producing bacteria

When a phytotoxin is suspected of contributing to disease, extraction of the compound from the producing organism is required. Knowledge of organic chemistry is required at this point since the ultimate goal will be to obtain or verify the structure of the toxin using mass spectrometry, NMR, or other analytical methodology. Reproduction of some aspect of the disease using purified compound is essential to proving the role of the phytotoxin in symptom development.

Bioassays for toxins produced by P. syringae

Some phytotoxins are antimicrobial and can be detected in bioassays using sensitive fungi or bacteria. For example, phaseolotoxin can be detected at picogram levels by growth inhibition of *Escherichia coli* K-12 (Staskawicz and Panopoulos, 1979), and tabtoxin has been detected in bioassays using toxin-sensitive bacteria or fungi (Gasson, 1980). Although the phytotoxin COR is not antimicrobial, it can be detected by its ability to induce chlorosis in a variety of plants; however, this assay is qualitative rather than quantitative. Völksch et al. (1989) have described a semiquantitative bioassay for COR in which a hypertrophic reaction on potato tubers is utilized to detect the toxin. This assay is sensitive, but variability can occur depending on the potato cultivar utilized and the age of the tuber.

Analytical methods for assessing toxin production

Quantitative chromatographic methods are available for detecting COR and tabtoxin (Barta et al., 1993; Bender et al., 1989). To facilitate genetic studies of COR biosynthesis, a rapid extraction and fractionation method for COR was developed which involves the direct extraction of organic acids from 0.5 ml of culture supernatant, a 9-min fractionation on a reverse-phase C_8 column in a gradient of acetonitrile and water, and quantitative detection at 208 nm (Palmer and Bender, 1993). The extraction can be performed in microcentrifuge tubes, takes approximately 3 min to complete, and accurately separates and quantifies CFA, COR, coronafacoylvaline (CFA-val), and coronafacoylisoleucine (CFA-ile). The availability of quantitative detection methods for COR and the two defined intermediates in the COR pathway (CFA and CMA) has greatly facilitated the analysis of mutant phenotypes (Bender et al., 1996).

Molecular detection of phytotoxins and toxin synthesis genes

The cloning of toxin gene clusters has facilitated the development of DNA probes and PCR primers for the identification of phytotoxin-producing strains of *P. syringae*. A DNA probe containing *ptx*, a gene required for phaseolotoxin production (Hatziloukas et al., 1995), was used to detect and identify *P. syringae* pv. *phaseolicola* from mixed cultures and diseased specimens (Schaad et al., 1989). Sets of oligonucleotide primers derived from the DNA probe were sensitive enough to detect *P. syringae* pv. *phaseolicola* in bean seed lots (Audy et al., 1996; Schaad et al., 1995; Tourte and Manceau, 1994). Recently, PCR primers based on the sequence of the *argK* gene, which encodes ROCTase, were used for detection of *P. syringae* pvs. *phaseolicola* and *actinidiae* (Mosqueda-Cano and Herrera-Estrella, 1997; Sawada et al., 1997). The PCR amplification procedure was applied directly to bacteria present in seed extracts and shown to be extremely sensitive for detection of *P. syringae* pv. *phaseolicola* (Mosqueda-Cano and Herrera-Estrella, 1997). Similarly, DNA probes and PCR primers from the COR biosynthetic gene cluster have proven useful for the detection of COR-producing pathovars of *P. syringae* (Bereswill et al., 1994; Cuppels et al., 1990).

Although DNA probes and PCR primers are both specific and sensitive detection methods, they do not indicate whether the phytotoxin is actively synthesized *in vitro* or *in planta*. Although serological methods are more attractive for this type of analysis, the phytotoxins discussed in this review are too small to be immunogenic and must be conjugated to a carrier protein which is large enough to generate antisera. Recently, Jones et al. (1997) constructed a COR-ovalbumin (OVA) conjugate where COR was linked to OVA at the free carboxyl group present on CMA. This hapten-conjugate was used to produce monoclonal antibodies (MABs) which were subsequently used in a competitive ELISA. One monoclonal line recognized COR and CFA-val equally well, and CFA-ile to a lesser extent. Furthermore, the MAB did not recognize CFA or CMA, the

nonphytotoxic intermediates in the pathway to COR. Since COR-producing *P. syringae* strains often synthesize coronafacoyl analogues, this monoclonal should be useful in detecting multiple coronafacoyl compounds simultaneously.

Engineering plants with phytotoxin resistance

Several strategies have been utilized to develop plants with resistance to phytotoxins. When phytotoxins are broadly antimicrobial, they are also frequently toxic to the producing organism. Consequently, one potential source of resistance is the phytotoxin-producer, *P. syringae*.

Transgenic plants with phaseolotoxin and tabtoxin resistance

argK, the gene encoding ROCTase, has been used to develop phaseolotoxin-resistant plants. Two laboratories have utilized *argK* as a source of phaseolotoxin resistance in tobacco (Hatziloukas and Panopoulos, 1992; de la Fuente-Martinez et al., 1992). Because OCTase is produced in the chloroplast, *argK* was targeted to this organelle by fusing the *argK* coding region to the transit peptide of the small subunit of ribulose bisphosphate carboxylase (de la Fuente-Martinez et al., 1992). Transgenic plants expressing ROCTase did not turn chlorotic when treated with phaseolotoxin and had no change in chlorophyll content. However, control tobacco plants which contained SOCTase turned chlorotic in response to phaseolotoxin and were reduced in chlorophyll content. Furthermore, control tobacco plants were systemically infected when inoculated with *P. syringae* pv. *phaseolicola*, but the transgenics expressing ROCTase showed a localized HR in response to infection. Therefore, a level of host resistance was expressed in the transgenics to both the pathogen and toxin (de la Fuente-Martinez et al., 1992). The next challenge will be to introduce *argK* into bean plants, the natural host for *P. syringae* pv. *phaseolicola*.

As mentioned previously, tabtoxin and TβL inhibit glutamine synthetase; consequently, tabtoxin-producing strains of *P. syringae* must protect themselves from these compounds. Anzai and co-workers (1989) reported on the isolation of a gene designated *ttr* for *t*ab*t*oxin *r*esistance; *ttr* conferred resistance to transgenic tobacco plants harboring this gene. Presumably, *ttr* functions to acetylate tabtoxin and TβL, and the acetylated forms of these toxins are nontoxic (Anzai et al., 1989). Other potential sources of tabtoxin resistance include the β-lactamases synthesized by *P. syringae* pv. *tabaci* and the enzyme which adenylates glutamate synthetase, rendering it insensitive to tabtoxin and TβL (Knight et al., 1986; 1987).

Selection for coronatine-resistance in Arabidopsis thaliana

Coronatine does not show antibiotic activity to prokaryotic cells, so COR-producers do not possess a resistance gene which could be utilized to develop transgenic plants with insensitivity to the toxin. However, one alternative strategy is to use mutagenesis to select coronatine-resistant (Cor^R) plants and then identify the target site in the mutants by map-based cloning. For example, the *coi1* mutant of *Arabidopsis*, which shows insensitivity to coronatine, was obtained by exposing seeds to mutagenic concentrations of ethylmethanesulfonate (Feys et al., 1994). The wild-type allele (*COI1*) was recently localized by mapped-based cloning, sequenced, and shown to contain leucine-rich repeats and a F-box motif, which suggests that the COI1 protein has a role in protein–protein interactions (Xie et al., 1998). Ultimately, *COI1* and the identification of related genes may facilitate the introduction of Cor^R genes into agronomically important host plants, such as tomato and soybean.

Conclusions

This review has focussed on three chlorosis-inducing phytotoxins produced by *P. syringae*. Like many of the gene clusters encoding antibiotic biosynthesis, phytotoxin genes are frequently clustered and often contain a resistance gene. Furthermore, the origins of phytotoxin gene clusters often show strong similarity to antibiotic biosynthetic gene clusters. For example, COR biosynthesis requires the cooperative activity of polyketide synthetases for the CFA moiety and a modified peptide synthetase for CMA assembly. Although considerable progress has been made in the cloning of phytotoxin gene clusters, much remains to be done, especially in the analysis of gene function and the regulation of toxin gene clusters. These areas are especially amenable to interdisciplinary studies and collaborations, particularly between chemists, biochemists, and geneticists.

Both tabtoxin and phaseolotoxin are strongly antimicrobial and function by inhibiting glutamine synthetase and OCTase, respectively. An understanding of the mode of action for these phytotoxins has opened up new avenues for the cloning of toxin resistance genes and their deployment in the development of resistant plants. Although the target site and mode of action remain unclear, efforts are underway to study these aspects of coronatine.

Characterization of the phytotoxin gene clusters discussed in this review has led to improved methods for the detection and diagnosis of bacterial diseases incited by *P. syringae*. Molecular diagnostic methods have been successfully used to detect *P. syringae* strains which produce phaseolotoxin and coronatine. Serological methods also have great potential for detection and would facilitate the detection of phytotoxins *in planta*.

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