Efflux Pump Gene Expression in *Erwinia chrysanthemi* Is Induced by Exposure to Phenolic Acids

Ramani S. Ravirala,¹ Ravi D. Barabote,² David M. Wheeler,³ Sylvie Reverchon,⁴ Ouwatha Tatum,⁵ Jeremy Malouf,⁵ Hui Liu,⁶ Leighton Pritchard,⁶ Peter E. Hedley,⁶ Paul R. J. Birch,⁶ Ian K. Toth,⁶ Paxton Payton,³ and Michael J. D. San Francisco¹

¹Department of Biological Sciences and Center for Biotechnology and Genomics, Texas Tech University, Lubbock 79409, U.S.A.; ²Department of Biological Sciences, University of California–San Diego, San Diego, U.S.A.; ³United States Department of Agriculture–Agricultural Research Service Cropping Systems Research Laboratory, Lubbock, TX 79415, U.S.A.; ⁴Unité de Microbiologie et Genetique, UMR CNRS-INSA-UCB 5122, 69622 Villeurbanne, France; ⁵School of Allied Health, Texas Tech University Health Sciences Center, Lubbock 79410, U.S.A.; ⁶Scottish Crop Research Institute, Dundee, Scotland

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Salicylic acid (SA) is an important signaling molecule in local and systemic plant resistance. Following infection by microbial pathogens and the initial oxidative burst in plants, SA accumulation functions in the amplification of defense gene expression. Production of pathogenesis-related proteins and toxic antimicrobial chemicals serves to protect the plant from infection. Successful microbial pathogens utilize a variety of mechanisms to rid themselves of toxic antimicrobial compounds. Important among these mechanisms are multidrug-resistance pumps that bring about the active efflux of toxic compounds from microbial cells. Here, we show that a combination SA and its precursors, *t*-cinnamic acid and benzoic acid, can activate expression of specific multidrug efflux pump-encoding genes in the plant pathogen *Erwinia chrysanthemi* and enhance survival of the bacterium in the presence of model as well as plant-derived antimicrobial chemicals. This ability of plant-pathogenic bacteria to co-opt plant defense-signaling molecules to activate multidrug efflux pumps may have evolved to ensure bacterial survival in susceptible host plants.

Additional keywords: *acrA*, bacterial resistance, *emrA*.

In response to microbial attack, plants activate defense responses that lead to induction of a broad spectrum of antimicrobial defenses (Van Loon 2000). These induced defenses are expressed not just locally but also in parts distant from the site of primary infection, thereby protecting the plant from spread of an infection and future attack. Induced resistance is regulated by a network of interconnecting signal transduction pathways in which salicylic acid (SA) is a key signaling molecule (Feys and Parker 2000; Pieterse and Van Loon 1999). SA-dependent resistance has been shown to be specific to microbial pathogens (Delaney et al. 1994; Wildermuth et al. 2001).

One of the earliest responses of plants to pathogen attack is the generation of an oxidative burst that can trigger hypersensitive cell death (Greenberg 1996). This hypersensitive response (HR) is associated with increased synthesis of lignin, callose,

and antimicrobial compounds such as phytoalexins that help to limit pathogen spread. The HR finally leads to the induction of systemic acquired resistance (SAR). The development of SAR results in a broad spectrum of resistance to subsequent infections (Dangl 1998; Klessig et al. 2000; Mauch-Mani and Slusarenko 1996). The HR also may be induced during plant responses to non-host-specific bacterial pathogens such as *Erwinia chrysanthemi* that utilize a type III secretion system to deliver proteins into the plant cytosol or the plant intercellular space (Alfano and Collmer 1997; Bauer et al. 1994). Plant cell wall fragments (oligogalacturonides), generated by the action of cell-wall-degrading enzymes, notably pectinases, produced by *E. chrysanthemi*, induce pathways of phenylpropanoid metabolism in plants generating phenolic acids such as SA and a wide array of antimicrobial proteins and chemicals, including quinones, flavonoids, phenolics, coumarins, terpenes, and alkaloids (Cowan 1999; Hahn et al. 1981; Hammerschmidt 1999; Ryan and Farmer 1991; Tenhaken et al. 1995).

SA is derived primarily from phenylalanine, with *trans*-cinnamic acid (TC) and benzoic acid (BA) serving as intermediates (Chong et al. 2001; Coquoz et al. 1998; Mauch-Mani and Slusarenko 1996; Ryan and Farmer 1991; Yalpani et al. 1993). Phenylalanine ammonia lyase (PAL) that catalyzes the first step in the pathway converting phenylalanine to TC has been shown to rise in pathogen-inoculated leaves continually for 18 h (Smith-Becker et al. 1998).

Although our understanding of *E. chrysanthemi* virulence factors and their regulation has become increasingly clear (Collmer and Keen 1986; Hugouvieux-Cotte-Pattat et al. 1996), we have a less complete understanding of how this bacterium survives in the plant following infection and how it tolerates the onslaught of plant antimicrobial chemicals. The importance of gene products involved in resistance to antimicrobial peptides, repair of oxidatively damaged proteins, protection from nitrosoative stress, and attenuation of host HR during infection have been demonstrated (Boccardo et al. 2005; El Hassouni et al. 1999; Lopez-Solanilla et al. 1998, 2001; Nachin et al. 2001; Okinaka et al. 2002). We have demonstrated that the outer membrane protein TolC which, together with multidrug efflux proteins, forms a continuous channel across the periplasm and both membranes, is essential for bacterial resistance to antimicrobial chemicals and survival in plant tissue (Barabote et al. 2003). Analysis of the *E. chrysanthemi*

themi genome shows that it contains homologs of *Escherichia coli* AcrAB and EmrAB efflux pumps to remove toxic antimicrobial chemicals from the cell and, recently, the importance of several efflux pumps has been demonstrated (Valecillos et al. 2006).

Here, we show that *E. chrysanthemi* efflux pump gene expression is upregulated in plant tissue and also in the presence of the phenolic acid signaling molecules. Additionally, tolerance of the bacterium to model and plant-derived antimicrobial agents is enhanced in the presence of these phenolic acids. These studies suggest that bacterial plant pathogens have evolved strategies to co-opt defense-related plant signaling chemicals to enhance their resistance to toxic plant chemicals and, consequently, to ensure survival in a hostile host environment.

RESULTS

Efflux pump gene expression is induced in plant tissue early in the infection process.

Annotation of the *E. chrysanthemi* 3937 genome sequence indicates the presence of genes encoding homologs of the *Escherichia coli* AcrAB and EmrAB efflux pumps (Glasner et al. 2006). These two pumps, as well as others, utilize a common outer membrane protein, TolC, that has been shown to be important for complete removal of toxins from the bacterial cell and is essential for survival of this bacterium in the host (Barabote et al. 2003). Expression of the *acrA* and *emrA* genes during the early stages of plant infection was monitored by luciferase or β -glucuronidase activity, respectively. Gene activity for both these pump-encoding genes was detected just 90 min following whitloof chicory leaf infections (Fig. 1A and B). Growth of bacteria over this 90-min interval increased approximately 2-fold and expression of *acrA::lux* was approxi-

mately 50-fold greater than the control. A protein bearing immunological identity to *Escherichia coli* AcrA with the predicted size of the *E. chrysanthemi* 3937 AcrA was observed to be induced 90 min following inoculation of *E. chrysanthemi* cells into whitloof chicory leaves (Fig. 2). These observations suggest that activation of efflux pump-encoding genes takes place before significant bacterial growth occurs in plant tissue and, presumably, before significant build up of antimicrobial chemicals occurs in plant tissue.

Phenolic acids enhance expression of the *acrA* and *emrA* efflux pump-encoding genes in *E. chrysanthemi*.

Previous studies have shown that SA and weak acids can stimulate efflux pump gene expression in enteric bacteria (Aleksun and Levy 1999; Randall and Woodward 2001; Rosner 1985). Because plants produce SA and other phenolic acids as part of the defense repertoire following infection by microbial pathogens, we decided to test the impact of these chemicals on efflux pump gene expression. Exposure of *E. chrysanthemi* cells for 90 min to phenolic acids at a concentration of 0.078 mM resulted in significant stimulation of *acrA* gene expression compared with the dimethyl sulfoxide (DMSO) control (Fig. 3A). Concentrations below and above 0.078 mM did not significantly induce *acrA* gene expression. At higher

Fig. 2. Immunoblot analysis using anti-AcrA (*Escherichia coli*) with *Erwinia chrysanthemi* 3937 whole-cell proteins following infection of plant tissue. Lane 1, *Escherichia coli* AcrA; (antiserum control); lane 2, *Erwinia chrysanthemi* (buffer control); lane 3, blank; lane 4, *E. chrysanthemi* cells recovered after 90 min, following inoculation into plant tissue.

Fig. 1. A. In vivo efflux pump expression of the *Erwinia chrysanthemi* 3937 *acr::lux* transcriptional fusion on plasmid pHSK728. Leaves of whitloof chicory were inoculated with 10^6 bacteria from an overnight culture. Measurements were made at 5 and 90 min after inoculation using a Kodak Image Station 440. **B.** In vivo efflux pump expression of the *E. chrysanthemi* 3937 carrying the *emr::uidA* transcriptional fusion on plasmid pBluescript. Leaves of whitloof chicory were inoculated with 10^6 bacteria from an overnight culture. β -Glucuronidase activity was detected in situ at 5 and 90 min post infection using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide as a substrate.

concentrations, the combination of phenolic acids appeared to be toxic and did not stimulate *acrA* gene expression. These observations suggest that *acrA* gene expression is highly sensitive to a very narrow range of phenolic acid concentrations. BA was the only phenolic acid that, when used alone, showed significant stimulation of *acrA* gene activity at concentrations of 0.156 and 0.313 mM compared with the DMSO control (Fig. 3B). Exposure to the other phenolic acids separately resulted in only modest increases in *acrA* gene activity (data not shown). The most potent inducer of *emrA* gene activity was found to be SA, which stimulated β -glucuronidase activity by approximately threefold at 0.313 mM.

To obtain a genome-wide perspective on the influence of the combination of phenolic acids and verify our efflux pump reporter gene expression observations, we utilized *E. chrysanthemi* 3937 microarray experiments. These experiments were conducted in triplicate using Agilent microarrays. Treatment with PA at a concentration of 0.078 mM for 90 min resulted in a significant ($P < 0.05$) increase (approximately 150 genes) or decrease (approximately 60 genes) over the DMSO control. Expression of three efflux pump-encoding genes (*acrA*, *emrA*, and one unknown efflux pump-encoding gene) was significantly upregulated (Table 1). Expression of genes encoding the other efflux pumps (approximately 29 in number) did not show a significant change in expression following this treatment.

The outer membrane protein-encoding gene *ompX* which plays a role in antibiotic and detergent (sodium dodecyl sulfate) resistance in *Escherichia coli* and *Yersinia pestis* (Otto and Hermansson 2004) also was significantly upregulated. Other genes encoding proteins involved in bacterial oxidative stress resistance, *osmC* and *gst*, also were significantly upregulated in these experiments. *Escherichia coli* lacking OsmC is hypersensitive to H_2O_2 and *tert*-butylhydroperoxide, indicating that the protein is involved either directly or indirectly in defense against oxidative stress (Conter et al. 2001). Glutathione-S-

Table 1. Antibiotic and stress resistance-related genes induced in *Erwinia chrysanthemi* 3937 following exposure to the combination of phenolic acids (salicylic acid, benzoic acid, and *trans*-cinnamic acid)

ASAP ID ^a	Gene	Annotation	<i>t</i> Test <i>P</i> value ^b
15971	<i>emrA</i>	Efflux pump	3.21E-04
19532	<i>acrA</i>	Efflux pump	7.73E-03
18909	<i>ompX</i>	Outer membrane protein	8.64E-03
17804	<i>osmC</i>	Osmotic stress	7.51E-06
17805	<i>gst</i>	Stress resistance	2.47E-09
16284	Unknown	Efflux pump	4.58E-02

^a ASAP database (Glasner et al. 2006).

^b Increases of average signal log ratio from three biological replicates were evaluated by GeneSpring software (*t* test, *P* value ≤ 0.05).

Fig. 3. In vitro *acrA::lux* response of *Erwinia chrysanthemi* 3937 exposed for 90 min to **A**, the combination of phenolic acids (salicylic acid, benzoic acid [BA], and *trans*-cinnamic acid) or **B**, BA alone. Statistically significant fold increases in gene expression were observed at the combination of phenolic acids at 0.078 mM ($P < 0.005$) and BA concentrations of 0.156 and 0.313 mM ($P < 0.05$). Measurements were made using a Turner-Roberts luminometer. An average of three separate experiments with error bars representing the standard error of the mean are presented. Phenolic acid treatment (□) and dimethyl sulfoxide solvent as control (■).

transferases catalyze the conjugation of hydrophobic substrates, such as drugs, herbicides, and insecticides, to glutathione (GSH) (Eaton and Bammler 1999). This conjugation increases their solubility and facilitates further metabolic processing.

Quantitative real-time reverse-transcriptase polymerase chain reaction analysis.

In order to validate our *in vivo* gene fusion and microarray observations, we utilized real-time quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR) using RNA isolated from *E. chrysanthemi* cells exposed to either PA (0.078 mM) or DMSO as a solvent control for 90 min. Experiments were performed using SYBR green with primers specific for *acrA*, *emrA*, *ompX*, and the 16S rRNA-encoding gene (Table 2). Reactions lacking the template served as a negative control. An approximately fivefold induction was observed with *emrA*, whereas *acrA* and *ompX* each were induced approximately fourfold (Fig 4). There was no notable change in expression for the 16S rRNA-encoding gene under these conditions. These data are in good agreement with our microarray and *in vivo* gene fusion studies.

Tolerance of *E. chrysanthemi* to model and plant antimicrobial agents is enhanced in the presence of phenolic acids.

To study whether the increase in efflux pump gene expression following exposure to phenolic acids actually translates into enhanced tolerance to antimicrobial agents, we employed a viable cell count assay. In these experiments, each antimicro-

Fig. 4. Quantitative real-time reverse-transcriptase polymerase chain reaction analysis in *Erwinia chrysanthemi* induced in response to the combination of phenolic acids (salicylic acid, benzoic acid, and *trans*-cinnamic acid). Fold change in the presence of the phenolic acids (0.078 mM) (□) or dimethyl sulfoxide control (■). Statistical analysis to describe the relative relationship between the test and control samples was carried out as previously described (Pfaffl 2001) using the 16S rRNA-encoding gene as an internal control. Results are means from three independent experiments.

Table 2. Oligonucleotide primers used in this study

Primer	Sequence 5'–3'	Application ^a	Product size (bp)
<i>acrA</i> F	GGAAGGCAGCGACGTCGG	RT-PCR	...
<i>acrA</i> R	TTTACGCCCAGTAATTCGGATT	RT-PCR	50
<i>emrA</i> F	CGCTCAAACGTACCCATATCG	RT-PCR	...
<i>emrA</i> R	CTGCGGCGGGAGACATAG	RT-PCR	50
<i>ompX</i> F	CCAGCGATGCTGGTTTGT	RT-PCR	...
<i>ompX</i> R	CGTTCTGAACCGGGTTGAAC	RT-PCR	50
16S F	CCAGCAGCCGCGTAAT	RT-PCR	...
16S R	TTTACGCCCAGTAATTCGGATT	RT-PCR	50

^a RT-PCR = reverse-transcriptase polymerase chain reaction.

bial agent was tested at a level just below the MIC. The colony count on agar medium following 12 h of exposure to the antimicrobial agent in the presence or absence of PA (0.078 mM) was used to determine the relative increase in tolerance. Our results indicate that growth in carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) or plumbagin (PB) was enhanced approximately two- or eightfold, respectively, in the presence of PA (Table 3). These observations provide relevance to enhanced efflux pump gene expression in the presence of phenolic acids.

DISCUSSION

Drug efflux pumps in bacteria are recognized as major contributors to bacterial survival. Mutations in efflux pump-encoding genes have been shown to sensitize bacteria to antimicrobial chemicals (Borges-Walmsley and Walmsley 2001; Nikaido 1996). Loss or impairment of efflux pump function also compromises the ability of pathogenic bacteria to cause disease or survive in their hosts. This has been observed in animal pathogens *Salmonella enteritidis* and *Vibrio cholerae* (Bina and Mekalanos 2001; Stone and Miller 1995) and in plant-associated bacteria *E. chrysanthemi*, *E. amylovora*, *Agrobacterium tumefaciens*, and *Rhizobium etli* (Barabote et al. 2003; Burse et al. 2004a,b; Gonzalez-Pasayo and Martinez-Romero 2000; Lopez-Solanilla et al. 1998, 2001; Palumbo et al. 1998; Valecillos et al. 2006).

Successful pathogens such as *E. chrysanthemi* that incite an HR and ensuing production of antimicrobial chemicals might sense preexisting and newly synthesized phenolic acids that serve in plant signaling. In healthy plants, conjugated BA pools decrease transiently after pathogen attack with concomitant synthesis of SA (Chong et al. 2001; Coquoz et al. 1998). The observation that the combination of phenolics acid or BA alone were the most powerful inducers of bacterial efflux pump gene expression suggests that bacterial defenses may be primed before plant antimicrobial agents are produced. Because

Table 3. Phenolic acids enhance antibiotic resistance in *Erwinia chrysanthemi*^a

Treatment	Antibiotic	Antibiotic + phenolic acids
CCCP	9.4×10^6	2.1×10^8
Plumbagin	2.0×10^5	1.7×10^7
Novobiocin	8.1×10^8	2.7×10^9
Berberine	1.9×10^8	1.5×10^9

^a Tolerance of *E. chrysanthemi* to antibiotics is enhanced following exposure to a combination of phenolic acids. Values indicate viable cell number per milliliter. Cells were grown in liquid culture and inoculated at 10^6 cell/ml into flasks containing subminimal inhibitory concentrations of the antimicrobial agents. Viable counts were determined after growth for 12 h at 30°C. Results represent an average of four separate experiments with three replicates each and indicate that there is a statistically significant enhancement of growth following treatment with phenolic acids (t test, *P* value ≤ 0.05). Inducer phenolic acids salicylic acid, benzoic acid, and *trans*-cinnamic acid were added at concentration of 0.078 mM each. CCCP = carbonyl cyanide *m*-chlorophenylhydrazine.

TC and BA are precursors of SA and, conceivably, are present together at an infection site, our observation of efflux pump gene induction by these chemicals together is interesting and relevant.

The *acrAB* operon in enteric bacteria encodes a periplasmic, membrane fusion protein, AcrA, and a membrane protein, AcrB (Murakami et al. 2002). This efflux pump functions together with the outer membrane protein TolC to bring about the rapid energy-dependant efflux of a wide variety of antimicrobial agents (Fralick 1996; Koronakis et al. 2000). The importance of components of this tripartite efflux pump has been demonstrated by mutational analysis in the plant pathogens *E. chrysanthemi* and *E. amylovora* (Barabote et al. 2003; Burse et al. 2004a,b; Valecillos et al. 2006). The EmrAB efflux pump has a relatively small substrate range when compared with the AcrAB pump in the family *Enterobacteriaceae*. Whereas the AcrAB pump expels bile salts, dyes, detergents, organic solvents, and antibiotics, the EmrAB pump substrate range is limited to a few ionophores and antibiotics (Borges-Walmsley and Walmsley 2001). Recent work also has demonstrated the contribution of members of the Emr family to drug resistance and phytopathogenicity in *E. chrysanthemi* (Valecillos et al. 2006).

Our observation of induction of *acrA* gene expression by phenolic acids was predicted based on previous studies with members of the family *Enterobacteriaceae*. Transcription of the global transcriptional activator MarA is derepressed in these bacteria following exposure to phenolics such as SA (Grkovic et al. 2001; Miller and Sulavik 1996). The repressor MarR has been shown to bind SA, the inducer of the *marAB* operon (Aleksun and Levy 1999; Aleksun et al. 2001; Rhee et al. 1998). Screening of the *E. chrysanthemi* genome sequence reveals the presence of many transcriptional regulators related to the MarR family and transcriptional activators belonging to the AraC/XylS family, to which MarA belongs (Glasner et al. 2006). One or more of these regulatory proteins may be responsible for *acrA* induction by these phenolic acids.

Induction of the *emrAB* operon in enteric bacteria is under control of EmrR, which belongs to the MarR family of transcriptional regulators. Derepression of the *emrAB* operon in members of the family *Enterobacteriaceae* by SA takes place by interaction with EmrR (Lomovskaya et al. 1995). The *E. chrysanthemi* genome contains a highly homologous *emrR* gene contiguous with the *emrAB* operon. Therefore, our observation of induction of *emrA* gene expression in the presence of phenolic acids is not unexpected. Both AcrA and EmrA are periplasmic proteins that functionally interact with integral membrane proteins (AcrB and EmrB, respectively) that are encoded in their cognate operons. We did not observe significant induction of *acrB* or *emrB* genes with phenolic acids in our microarray experiments. It is possible that differences in mRNA stability or the presence of regulatory structures within intercistronic regions of the operon encoding render these transcripts to be differentially expressed, as has been previously noted (Barabosa and Levy 2000).

The concentrations of phenolic acids at which we observed significant efflux pump gene expression in vitro are well within the range of these chemicals calculated to be present in infected plant tissue. The reported concentrations of SA in plants vary from 150 to 300 μ M in leaf tissue to 300 μ M in roots and 600 μ M in phloem exudates (Smith-Becker et al. 1998; Yalpani et al. 1993). SA is synthesized from pools of conjugated BA, which then is converted to its aglycone by BA 2-hydroxylase (Leon et al. 1993). BA is formed from TC, which is derived from phenylalanine by PAL (Coquoz et al. 1998; Ribnicky et al. 1998; Yalpani et al. 1993). The first step in the pathway converting phenylalanine to TC has been

shown to rise in pathogen-inoculated leaves continually for 18 h following infection (Smith-Becker et al. 1998).

In *Escherichia coli*, exposure to phenolic compounds results in enhanced expression of genes belonging to the *mar* operon (Cohen et al. 1993; Martin and Rosner 2002, 2004; Pomposiello et al. 2001). In addition, general stress response genes such as *dnaK* (heat shock), *sulA* (inhibition of cell division), *dps* (DNA-binding iron chelator), *katE* (catalase), and *rpoS* (global regulator) also are upregulated in the presence of phenolic compounds (Pomposiello et al. 2001). Our observations in *E. chrysanthemi* indicate that the primary response to phenolic acid exposure appears to be more specific and limited to antimicrobial agent and related stress resistance (Table 1), and does not include the general stress response genes. These differences may be due to the fact that studies with *Escherichia coli* used 5 mM SA whereas, with *E. chrysanthemi*, we used a concentration of 0.078 mM for each of the three phenolic acids.

Our data suggest that successful microbial pathogens like *E. chrysanthemi* can co-opt plant signaling chemicals such as phenolic acids that enhance tolerance to toxic plant antimicrobial chemicals. Studies on specific interactions between plant-derived phenolic acids and regulatory proteins in *E. chrysanthemi* currently are underway to allow us to more fully understand the complex signaling that occurs between pathogen and host.

MATERIALS AND METHODS

Bacterial strains and growth of cells.

E. chrysanthemi 3937 was used in these studies. Luria-Bertani (LB) using only half the amount of NaCl was used as the growth medium for all experiments. Bacterial cells were grown in liquid culture and inoculated at 10^6 cells/ml into flasks for the in vitro studies. Cultures were grown for 18 h at 30°C and shaken, and viable cell counts were determined by serial dilution and plating. PB, CCCP, SA, BA, and TC were purchased from Sigma-Aldrich (St. Louis). Minimal inhibitory concentrations (MICs) of the drugs tested were determined using twofold dilutions of the chemicals in LB growth medium in microtiter plates as previously described (Barabote et al. 2003; Fralick 1996). The MIC is defined as the lowest concentration of a drug that prevented bacterial growth. Sub-MIC concentrations of CCCP and PB were 6 and 30 μ g/ml, respectively. All in vitro induction experiments utilized LB plus the inducer PA (a combination of phenolic acids SA plus TC plus BA, each at 0.078 mM) unless stated otherwise. As a control for these experiments, the solvent DMSO was used at the same volume as the inducers.

Construction of reporter gene fusions.

An operon (transcription) fusion of *acrA* with the entire *lux* operon was constructed in wild-type *E. chrysanthemi* using pHSK728 carrying a promoterless *lux* operon downstream of a multiple cloning site (Shen et al. 1992). The PCRs using *E. chrysanthemi* 3937 chromosomal DNA as template were carried out using Promega buffers and *Taq* polymerase (Fisher), dNTPs (Otsu, Tokyo), and primers (Center for Biotechnology and Genomics, Texas Tech University) at the following final concentrations: 1 $\times$ reaction buffer, 1.5 mM MgCl₂, 200 μ M NTPs, 100 pM primers, and 1 unit of *Taq* polymerase. All reactions were carried out on an MJ Research PTC-100 machine (Waltham, MA, U.S.A.). Products were electrophoresed on 0.8% agarose gels and purified using the QIAEXII purification kit (Qiagen, Valencia, CA, U.S.A.). Fragments were sequenced to confirm identity at the Center for Biotechnology and Genomics, Texas Tech University, and cloned into the pTOPO PCR2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.).

The promoter-containing fragment then was excised as an *EcoRI* fragment and cloned into pHSK728. The orientation of the cloned fragment was confirmed by nucleotide sequencing. The *emrA::uidA* reporter fusion was constructed in pBluescript (chloramphenicol-resistant)) following insertion of an *uidA::npt* cassette into the unique *EcoRI* site located in *emrA* to yield plasmid pSR2826.

Efflux pump gene expression measurements.

An overnight culture of approximately 10^6 cells carrying *acrA::luxAB* or *emrA::uidA* fusions was added in a final volume of either 0.2 ml (in microtiter plates) or 1 ml of LB in tubes containing the appropriate inducers. Cells were incubated at 30°C for 90 min. For luciferase activity, 50- μ l aliquots of cells were pipetted into 96-well white Costar plates and recorded using a Microplate Luminometer (Turner BioSystems, Sunnyvale, CA, U.S.A.). For β -glucuronidase activity, cells were washed once with 50 mM potassium phosphate (pH 7.0), and 25 μ l of toluene was added and allowed to evaporate after vortexing. The substrate p-nitrophenyl β -D-glucuronide was added at a final concentration of 3 mM and incubated at 37°C for 30 min. The reaction was stopped with an equal volume of 0.5 M NaOH. The absorbance was measured at 405 nm using a Beckman DU 530 Spectrophotometer.

In planta efflux pump gene expression.

Heads of witloof chicory (*Cichorium intybus* L., Belgian endive) were purchased from a local supermarket. Overnight-grown cultures of appropriate bacterial strains were centrifuged and washed twice with 5 mM morpholino-ethanesulfonic acid, pH 6.5, and resuspended in the same buffer. Chicory leaves were inoculated by introducing 10 μ l (10^6 cells) of inoculum into a small wound created on the inner surface of each leaf using a sterile toothpick. Leaves were maintained in a moist chamber at 30°C for 90 min (Barabote et al. 2003). For *acrA::lux*, light was monitored using the Kodak Image station 440CF. For the in situ β -glucuronidase enzyme assay, 10 μ l of toluene was added to the infection site to permeabilize cells and allowed to evaporate, and 10 μ l of 40 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide was added to facilitate color development.

Immunoblot analysis.

Overnight cultures of *E. chrysanthemi* 3937 were centrifuged and washed twice in sterile phosphate buffered saline (PBS), pH 7.4, and resuspended in same buffer. Witloof chicory leaves were inoculated with 10^6 cells and incubated at 30°C for 90 min (Barabote et al. 2003). As a control, cells were incubated in sterile PBS for 90 min at 30°C. Cells were extracted from the leaf and optical density at 600 nm (OD_{600}) measured to normalize cell number with the control. Immunoblot analysis was performed using *Escherichia coli* anti-AcrA rabbit antiserum following protein separation on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transfer to polyvinylidene difluoride membranes according to standard procedures (Sambrook et al. 1989).

Microarray experimental design and *E. chrysanthemi* 3937 coding sequence-specific oligonucleotide probe use.

Flasks with 15 ml of prewarmed LB were inoculated with *E. chrysanthemi* cells at approximately 10^6 cells/ml from an overnight culture, grown, and shaken at 150 rpm at 30°C to midexponential growth (OD_{600} of 0.4) before addition of phenolic acids (0.078 mM each of SA, BA, and TC) (PA) or DMSO as a control. RNA was harvested from these cultures after 90 min as described below. For the microarray, RNA was prepared from three replicates of each inducer and control cultures.

The *E. chrysanthemi* 3937 coding sequences and a subset of the intergenic regions were selected and an initial set of candidate oligonucleotide probes designed using ArrayDesigner 1.17 (Premier Biosoft Int., Palo Alto, CA, U.S.A.). For all consecutive runs, the parameters were kept with constant values, as follows: Hairpin MaxDG was 5, SelfDimer MaxDG was 6, and Run/Repeat (dinucleotide) maximum length was 5. The final probes of 3937 were spotted using inkjet array technology to generate a 60-mer *E. chrysanthemi* 11K customer array (Agilent, Inc., Santa Clara, CA, U.S.A.), which contained 5,862 probes from the *E. chrysanthemi* genome (the entire open reading frame complement of Ech 3937), 20 Lucidea spikes (Lucidea Universal ScoreCard, Amersham Biosciences, Little Chalfont, U.K.), and internal controls. Two reciprocal (dye-swap) hybridizations were performed for normalization and replication purposes. Most of the probes were spotted twice onto a single array.

cDNA synthesis, labeling, and hybridization.

Total RNA from cells grown in LB plus PA or DMSO was isolated using a Qiagen RNA extraction Kit (Qiagen). RNA was quantified using a NanoDrop (NanoDrop, Wilmington, DE, U.S.A.) and quality checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). Total RNA (12 μ g) was used for cDNA synthesis, using SuperScript II (Invitrogen) following the manufacturer's instructions. cDNA, carrying aminoallyl-dUTP (Ambion, Austin, TX, U.S.A.), was labeled by Cy3 or Cy5 and purified by Qiagen MinElute columns. The labeling efficiency was estimated using NanoDrop. Equivalent amounts of cDNA (control and sample) labeled with Cy3 or Cy5 were mixed with 25 μ l of control target and 105 μ l of 2 $\times$ hybridization buffer (Agilent Technologies) and then assembled in a chamber for hybridization. Hybridization was carried out for 17 h at 60°C in an Agilent oven. After hybridization, the slides were washed at room temperature, then dried by spin. Arrays were scanned using an ArrayWoRx Auto scanner (Applied Precision, Inc., Issaquah, WA, U.S.A.) with various exposure settings for Cy3 (595 nm) and Cy5 (685 nm) at 9.756- μ m resolution, generating separate TIFF images. Intensity data were acquired from images using SoftWoRx Tracker software. Images were imported and aligned with probe position (Agilent GAL file) using analyzerDG software automated and manual grid alignment features (SoftWoRx Tracker). Median signal spot and individual median background intensity values were extracted for each wavelength and imported into GeneSpring (v. 7.2; Agilent), whereby replicate spots in each array were averaged. Background-subtracted signals were corrected for unequal dye incorporation or unequal load of labeled product. The algorithm consisted of a rank consistency filter and normalization using the locally weighted linear regression (LOWESS) method. Saturated spots and the spots showing a reference signal lower than background were excluded from subsequent analyses. Based on standard deviation calculations, probes (genes) having ≥ 1.5 or ≤ 0.66 final sample/control (S/C) ratios were selected as statistically significant sample upregulated or sample downregulated. An increase or decrease of average signal log ratio from three biological replicates were evaluated by the GeneSpring software as significantly changed (*t* test, *P* value ≤ 0.05).

Quantitative real-time RT-PCR analysis.

RNA was isolated from cells induced with PA or DMSO as solvent control as described above. Reverse-transcriptase reactions were performed in a final volume of 100 μ l, using random hexamers with the High Capacity cDNA Synthesis Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, U.S.A.). Substrate RNA (5 μ g) was

used for each reverse-transcription reaction. Reaction mixtures were incubated at 25°C for 10 min followed by incubation at 37°C for 120 min. Specific primer pairs were designed with the ABI PRISM Primer Express software (Applied Biosystems) (Table 2). The 16S rRNA gene was chosen as an internal standard.

Real-time RT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Primer pairs at a final concentration of 0.6 µM were used to amplify a 50-bp fragment for all three genes. Change in fluorescence emission was detected and analyzed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Additional controls included omission of the reverse transcriptase to measure the extent of residual genomic DNA contamination, melting curve analysis, and use of a no-template control for each primer pair to measure interference from primer dimer formation. Three replicates were performed for each reaction. A standard curve was generated using two-fold dilutions of a known amount of the target gene as a template. PCR was performed using the following conditions: 1 cycle of 10 min at 95°C and 40 cycles of 94°C for 10 s, 55°C for 20 s, and 72°C for 60 s. To allow comparison of the relative expression levels of each gene, statistical analysis was carried out as previously described (Pfaffl 2001) using the 16S rRNA-encoding gene as an internal control. This method requires verification of the efficiency of the PCR parameters and conditions used. To accomplish this, the efficiency of dye incorporation levels that resulted upon PCR with each primer set and a standardized amount of template DNA were determined using the formula $E = 10^{(-1/\text{slope})}$. These values then were used to normalize the results. The relative quantification of the genes of interest versus the 16S rRNA encoding reference gene was accomplished using the following formula:

$$\text{Ratio} = ([E_{\text{target}}]^{\text{Ct control} - \text{Ct sample}}) / ([E_{\text{ref}}]^{\text{Ct control} - \text{Ct sample}})$$

where Ct control is the mean cycle threshold (Ct; an arbitrary baseline fluorescence at which exponential amplification of gene of interest begins) of the control sample, Ct sample is the mean Ct of the test sample, E_{target} is efficiency of the production of target gene transcript, and E_{ref} is efficiency of production of the reference gene transcript. The Ct values obtained from the Q-RT-PCR were confirmed using a paired *t* test before application to the above formula.

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