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Genome-Wide Identification of Plant-Upregulated Genes of *Erwinia chrysanthemi* 3937 Using a GFP-Based IVET Leaf Array

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A green fluorescent protein-based in vivo expression technology leaf array was used to identify genes in *Erwinia chrysanthemi* 3937 that were specifically upregulated in plants compared with growth in a laboratory culture medium. Of 10,000 *E. chrysanthemi* 3937 clones, 61 were confirmed as plant upregulated. On the basis of sequence similarity, these were recognized with probable functions in metabolism (20%), information transfer (15%), regulation (11%), transport (11%), cell processes (11%), and transposases (2%); the function for the remainder (30%) is unknown. Upregulated genes included transcriptional regulators, iron uptake systems, chemotaxis components, transporters, stress response genes, and several already known or new putative virulence factors. Ten independent mutants were constructed by insertions in these plant-upregulated genes and flanking genes. Two different virulence assays, local leaf maceration and systemic invasion in African violet, were used to evaluate these mutants. Among these, mutants of a *purM* homolog from *Escherichia coli* (*purM*::Tn*5*), and *hrpB*, *hrcJ*, and a *hrpD* homologs from the *Erwinia carotovorum hrpA* operon (*hrpB*::Tn*5*, *hrcJ*::Tn*5*, and *hrpD*::Tn*5*) exhibited reduced abilities to produce local and systemic maceration of the plant host. Mutants of *rhiT* from *E. chrysanthemi* (*rhiT*::Tn*5*), and an *eutR* homolog from *Salmonella typhimurium* (*eutR*::Tn*5*) showed decreased ability to cause systemic invasion on African violet. However, compared with the wild-type *E. chrysanthemi* 3937, these mutants exhibited no significant differences in local leaf maceration. The pheno type of *hrpB*::Tn*5*, *hrcC*::Tn*5*, and *hrpD*::Tn*5* mutants further confirmed our previous findings that *hrp* genes are crucial virulence determinants in *E. chrysanthemi* 3937.

Additional keywords: oxidative stress, phosphotransferase system, transport of oligogalacturonides, type III secretion system.

Erwinia chrysanthemi is an opportunistic necrotrophic pathogen that causes soft rot, wilts, and blight diseases on a wide range of plant species. Like other many enteric pathogens, such as *Escherichia coli* O157:H7 and *Yersinia pestis*, *Erwinia chrysanthemi* cells do not appear to invade host cells internally in the pathogenic phase. They remain in the intercellular spaces of infected plant tissue and use several secretion systems to inject virulence factors into host cells. In addition to causing local disease, the bacteria may enter vascular elements of infected plants, thereby moving rapidly through the host (Chatterjee et al. 2000; Collmer and Keen 1986; Expert 1999; Hugouvieux-Cotte-Pattat et al. 1996; Perombelon and Kelman 1980).

Many virulence determinants have been discovered in *E. chrysanthemi*, including the well-studied extracellular enzymes such as pectate lyase, pectinase, and cellulase; siderophore-dependent iron uptake systems, *sap*, and *msrA* gene (Expert 1999; Franza and Expert 1991; Franza et al. 1999; Hassouni et al. 1999; Lopez-Solanilla et al. 2001; Tardy et al. 1997). Virulence gene expression in *E. chrysanthemi* is a highly regulated phenomenon affected by a variety of parameters, including temperature, pH, iron levels, growth phase, and population density. This organism uses complex regulation strategies for virulence gene expression and possesses well-known sophisticated sensing systems such as ExpI/ExpR quorum sensing and two-component signal transduction pathways to sense and respond to environmental signals. It also utilizes transcriptional regulators such as cyclic AMP receptor protein (CRP), plant-inducible regulator (Pir), KdgR and PecS, and the RsmA/*rsmB* post-transcriptional regulatory system to coordinate virulence gene expression (Chatterjee et al. 2000; Franza et al. 1999; Hugouvieux-Cotte-Pattat et al. 1996; Nachin and Barras 2000; Nassar and Reverchon 2002; Nasser et al. 1994, 1998). Recently, the type III secretion system also has been reported to play a role in pathogenicity (Bauer et al. 1994, 1995; Lopez-Solanilla et al. 2001; Yang et al. 2002).

Virulence genes of pathogens can be divided into class I, class II, and class III categories according to Wassenaar and Gaastra (2001). Class I genes are called "true virulence genes", class II genes include global or specific regulators that regulate the activities of the class I genes, and class III virulence genes compromise host defense, cytoskeletal structure, and intracellular signaling, or encode other devices required for the pathogen to cope effectively with the nutrition-limited and generally hostile environment found within hosts. The distinction between class III virulence genes and housekeeping

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genes may be blurred. One major distinction is that, unlike true housekeeping genes, class III virulence genes are expected to be upregulated in the host environment compared with growth of the pathogen on laboratory culture media.

Unlike other host-specific plant bacterial pathogens, *E. chrysanthemi* is an opportunistic plant pathogen and attacks many different species of hosts. It is especially important to identify class III virulence genes in this unique bacterium because class III genes may facilitate *E. chrysanthemi* infection and colonization in a wide variety of plant hosts (Okinaka et al. 2002; Yang et al. 2002). Our results with microarray and mutagenesis experiments to study host upregulated genes of *E. chrysanthemi* 3937 on African violet have revealed several new class III virulence genes in the bacterium (Okinaka et al. 2002). Bacterial genes involved in virulence are likely to be plant inducible; therefore, instead of simply looking at type III secretion genes by sequence analysis and *hrp* functional assays (Boch et al. 2002; Fouts et al. 2002; Okinaka et al. 2002), we intend to identify diverse classes of virulence gene by using green fluorescent protein (GFP)-based in vivo expression technology (IVET) leaf array.

Beyond identification of virulence genes in *E. chrysanthemi* 3937, a broader understanding of host-regulated genes in this bacterium during the infection process is needed. Until now, various plant-regulated virulence genes of *E. chrysanthemi* 3937 have been identified (Expert 1999; Franza and Expert 1991; Franza et al. 1999; Hassouni et al. 1999; Lopez-Solanilla et al. 2001; Tardy et al. 1997), but little information is available on general host upregulated genes of the bacterium at a genomic level. In many infected plant tissues, a common shift in metabolic pattern, including activation of peroxidase and accumulation of an array of secondary metabolic substances (phenolics, flavonoids, coumarins, steroids, and so on) was observed (Goodman et al. 1986). As a consequence, many bacterial genes involved in growth inside of the plants might not necessarily be virulence genes, and altered global gene expression patterns are expected as the bacteria tailor their gene expression to adapt themselves inside of host plants (Okinaka et al. 2002). To broaden our understanding in this area, a stable GFP-based IVET leaf assay technique was introduced in this study to identify genes in *E. chrysanthemi* 3937 that are upregulated in a spinach host plant. Combining with the genome analysis database ASAP (a systematic genome package for community analysis of genomes) (Glasner et al. 2003), 10,000 individual IVET clones of *E. chrysanthemi* 3937 were screened for host upregulated genes and the identified genes were assigned to seven categories on the basis of their predicted cellular or physiological functions. Several plant upregulated genes were further selected and their roles in virulence on a plant host were investigated by two different assays, local leaf maceration and systemic invasion, on African violet. Several mutants of *E. chrysanthemi* 3937 caused local maceration but had reduced ability to cause systemic infection of the plant host.

RESULTS

IVET screening plant-upregulated genes.

The validity of our promoter-probe screening was tested with promoters of known virulence genes *hrpG*, *hrpN*, and *pelD*. Using a promoter-probe vector, pPROBE-AT (Miller and Lindow 1997), as a transcriptional reporter, a weak GFP fluorescence was observed from wild-type *E. chrysanthemi* 3937 cells containing promoter *hrpN* (pPROBE-AT::P*hrpN*) and *pelD* (pPROBE-AT::P*pelD*) grown in Luria-Bertani (LB) broth (Table 1). No to very weak expression of GFP was observed from *E. chrysanthemi* 3937 cells containing plasmid pPROBE-AT::P*hrpG* grown in LB broth. All three promoters of *hrpG*, *hrpN*, and *pelD* were found to be upregulated and produced elevated green fluorescent light in spinach leaves. No GFP expression was observed in wild-type *E. chrysanthemi* 3937 cells that contained the pPROBE-AT vector alone, or constructs pPROBE-AT::P*hrpG*op, pPROBE-AT::P*hrpN*op, and pPROBE-AT::P*pelD*op, in which the promoter sequences were in the opposite orientation with respect to the promoterless GFP promoter. Further verification of the IVET screening system was done by constructing a microarray clone ECH205 (*Bacillus subtilis* peptide synthase homolog, plant/culture ratio 5.4) (Okinaka et al. 2002) promoter region into pPROBE-AT vector as described above, and upregulated expression of GFP was observed in spinach leaves.

Plant upregulated clones of *E. chrysanthemi* were visually distinguished by a fluorescent microscope. To insure the consistency of our IVET screening, only the *E. chrysanthemi* library clones leading to bacterial fluorescence of similar or higher intensity as pPROBE-AT::P*hrpG*, and pPROBE-AT::P*hrpN* in leaves and no or low fluorescence in LB broth, were recognized as plant upregulated clones. The library clones showed minor differences in fluorescence signal within LB broth and plant leaf discs were eliminated because of the limitation of this method.

A library of 1.5-kb fragments of genomic DNA of wild-type *E. chrysanthemi* 3937 was ligated into the promoter-probe vector, pPROBE-AT. From 10,000 clones screened in spinach leaves, 61 plant-up-regulated clones of *E. chrysanthemi* 3937 were identified (Table 2). These upregulated clones were divided into seven functional categories, including metabolism (20%), information transfer (15%), regulation (11%), transport (11%), cell processes (11%), and transposases (2%). The function of the remaining 30% is unknown.

Among these plant-upregulated genes, several known or new putative virulence factor homologues of *Erwinia* spp. and other pathogens were identified. These include the type III secretion genes *hrpA* and *hrpB*, which also have been found in *Pseudomonas syringae* by the IVET method (Boch et al. 2002). The *dspE* homologue, observed for the first time in *E. chrysanthemi* 3937, is an essential pathogenicity factor that is secreted through the type III secretion pathway in *E. amylovora* (Bogdanove et al. 1998; Gaudriault et al. 1997). A potential *hrp* box, the consensus binding site sequence of the alternative sigma factor *hrpL*, was found upstream of the *hrpA* (5′-GGAACCACCTCGCATTATCTCCTACTTA-3′) and *dspE* genes (5′-GGAACCGGCGGCGGCGGCATACCACTCA-3′) of *E. chrysanthemi* 3937. In addition, genes involved in cell wall degradation and sugar transport also were identified as upregulated in spinach. These included type II secretory component (*out* genes), oligogalacturonate lyase (*ogl*), and a rhamnogalacturonide transporter (*rhiT).* Peptide methionine sulfoxide reductase (*msrA*), which repairs oxidized proteins and protects *E. chrysanthemi* cells from oxidative stress in host environments (Hassouni et al. 1999), also was found to be upregulated in our assay. In addition, we found that the indigoidine gene (*indA*), conferring resistance to oxidative stress (Reverchon et al. 2002), also was upregulated in the plant hosts.

Several regulatory genes were found to be upregulated in the host plant from our IVET assay. These include the LysR-family transcriptional regulatory protein MetR, the AraC-family regulatory proteins EutR and XylR, the IclR-family transcriptional regulator SrpS, the phosphate regulon transcriptional regulatory protein PhoB, the transcription factor RelB, and the cyclic AMP receptor protein CRP. CRP is a main activator of pectinolysis genes in *E. chrysanthemi* 3937 (Reverchon et al. 1997). Although we failed to recover iron uptake genes previously reported in *E. chrysanthemi* 3937 (Expert 1999;

Franza et al. 1999), several new putative iron uptake genes (*yfeA*, *hmuS/hmuT/hmuU*) were identified from our IVET assay. Several putative xenobiotic resistance genes (*bacA* and *crcB*), chemotaxis signaling related genes (*cheR* and *trg*), and a phosphotransferase system (PTS) gene (*nagE*) were found to be plant upregulated. In addition, we discovered several putative exported proteins with unknown function that were plant upregulated in the IVET assay. Five genes (ASAP IDs 14870, 16412, 18371, 19359, and 19592) were chosen as the representative of transporter, metabolism, regulator, and type III secretion pathway, respectively, for the future study.

Plant-upregulated gene knockout and virulence assays.

Using the in vitro Tn*5* insertional mutagenesis strategy, we constructed mutations in five plant upregulated genes and five adjacent open reading frames (ORFs) that might contribute to virulence. ASAP IDs 16412, 14870, 18371, 19592, and 19359 were plant upregulated genes identified in IVET assay. ASAP IDs 18369 and 18373 were genes flanking 18371. ASAP IDs 19590, 19588, and 19586 were genes flanking 19592. Mutants of *E. chrysanthemi* 3937 were considered to have reduced local leaf maceration ability when their pathogenicity index (PI) values were <1. Mutants *hrpB*::Tn*5*, *hrcJ*::Tn*5*, and *hrpD*::Tn*5* of *E. chrysanthemi* 3937, with PI values of 0.019, 0, and 0.016, respectively, exhibited lower local leaf maceration in African violet (Fig. 1; Table 3). In addition, they showed reduced ability to cause systemic invasion in African violet (Fig. 2). Although the ability to produce local maceration was not significantly altered for the *rhiT*::Tn*5* and *eutR*::Tn*5* mutants of *E. chrysanthemi* 3937, systemic invasiveness was impaired in both. Seven days after inoculation, 8 of 10 plants inoculated with wild-type *E. chrysanthemi* 3937 developed systemic invasion, while 3 and 4 of 10 plants developed similar symptoms in the *eutR*::Tn*5* and *rhiT*::Tn*5* mutants, respectively. Only 1 of 10 African violet plants developed systemic maceration symptoms in the *hrpB*::Tn*5* mutant. Using the statistical Fisher's exact test, there was a significant difference in systemic maceration capac-

Table 1. Strains, plasmids and DNA primers used in this study

Strains, plasmids, primers	Characters or sequences (5′ to 3′)[a]	Reference or source
Erwinia chrysanthemi strains		
3937	Wild type	Hugouvieux-Cotte-Pattat, N.
3937 *upp*::Tn*5*	Tn*5* Insertional mutant of *upp* homolog, Km^r	This work
3937 *rhiT*::Tn*5*	Tn*5* Insertional mutant of *rhiT*, Km^r	This work
3937 *uraA*::Tn*5*	Tn*5* Insertional mutant of *uraA* homolog, Km^r	This work
3937 *eutR*::Tn*5*	Tn*5* Insertional mutant of *eutR* homolog, Km^r	This work
3937 *purM*::Tn*5*	Tn*5* Insertional mutant of *purM* homolog, Km^r	This work
3937 *hrpB*::Tn*5*	Tn*5* Insertional mutant of *hrpB* homolog, Km^r	This work
3937 *hrcJ*::Tn*5*	Tn*5* Insertional mutant of *hrcJ* homolog, Km^r	This work
3937 *hrpD*::Tn*5*	Tn*5* Insertional mutant of *hrpD* homolog, Km^r	This work
3937 *pup2D*::Tn*5*	Tn*5* Insertional mutant of ECH19586, Km^r	This work
3937 *ECH16413*::Tn*5*	Tn*5* Insertional mutant of ECH16413, Km^r	This work
Plasmids		
pPROBE-AT	Promoter-probe vector, Ap^r	Miller and Lindow 1997
pGEMT-Easy	PCR cloning vector, Ap^r	Promega
pPROBE-AT: P*hrpG*	0.65-kb DNA fragment in pBROBE-AT containing *hrpG* promoter	This work
pPROBE-AT::P*hrpN*	0.5-kb DNA fragment in pBROBE-AT containing *hrpN* promoter	This work
pPROBE-AT::P*pelD*	0.6-kb DNA fragment in pBROBE-AT containing *pelD* promoter	This work
pPROBE-AT::P*hrpG*op	The transcription orientation of *hrpG* promoter is opposite to GFP reporter	This work
pPROBE-AT::P*hrpN*op	The transcription orientation of *hrpN* promoter is opposite to GFP reporter	This work
pPROBE-AT::P*pelD*op	The transcription orientation of *pelD* promoter is opposite to GFP reporter	This work
Primers		
pPBROBE-AT For	ACTGCCAGGAATTGGGGATCGGAAG	…
pPBROBE-AT Rev	AGTTCTTCTCCTTTACTCATA	…
hrpG Forward	GTCGACGGATAACCCGCAAAAACCGG	…
hrpG Reverse	TCAGTTGAAGTCATTGATGATGGCCTTC	…
hrpN Forward	TCGCTCGTCGTTATCAGCAG	…
hrpN Reverse	TCAGTTTATCCACGCTGGAACC	…
pelD Forward	CGGTAAACTGTAATTTCCCGCCCAGCG	…
pelD Reverse	TCATCGATGTAGCGATGATGGCTGCC	…
Tn*5* IR-F	GGTTGAGATGTGTATAAGAGACAG	…
Tn*5* IR-R	CCAACTCTACACATATTCTCTGTC	…
CH205 Forward	AAGCTTGTCAGTCAGGCAGGACAA	…
CH205 Reverse	GGATCCTGGAGTCTGGGTGAAGT	…
PUP2 Forward	ACCTTACGCCACACGATCGG	…
PUP2 Reverse	CGGAACCACCTCGCATTATC	…
PUP5 Forward	CTTGAGCTCGATCGCCA	…
PUP5 Reverse	TCAGCACGAATACCACGT	…
PUP5 Mutation Forward	GATGGCGTCCAACCTGAT	…
PUP5 Mutation Reverse	AGAGAAGAACGTGGCGAT	…
PUP16 Forward	TCATGACGGCTGACGTTG	…
PUP16 Reverse	TGGCTTCGTAGGTCAGCA	…
PUP16 Mutation Forward	CGATGTCAGTACCAAGCG	…
PUP16 Mutation Reverse	GGCAACGTCAGCCGTCAT	…
PUP21 Forward	CATGGGCCGCGTAGTCGATA	…
PUP21 Reverse	CGCCCTGGTGAAACAACAGA	…
PUP29/30 Forward	GGTCAGGAACATGGTCAC	…
PUP29/30 Reverse	GACACCTGACTGACAGGT	…
PUP29/30 Mutation Forward	TATCTTGTGCCCTTTGGC	…
PUP29/30 Mutation Reverse	GGGCGTAATGTTTCTGGT	…

[a] Km^r = kanamycin resistance and Ap^r = ampicillin resistance.

ity between the wild-type bacterium and the *eutR*::Tn*5* (P = 0.032), *hrpB*::Tn*5* (P = 0.02), and *rhiT*::Tn*5* (P = 0.075) mutants. The *purM*::Tn*5* mutant is an auxotroph and was not able to grow on M9 minimum medium. No significant reduction of virulence was observed in the other Tn*5* mutants constructed.

DISCUSSION

Recently, microarray gene expression technology was used to identify gene expression profiles of organisms in different environments (DeLisa et al. 2001; Okinaka et al. 2002; Wei et al. 2001). However, several limitations (e.g., low amount of available RNA from target cells and the presence of contaminating organisms in samples) still deter the usage of this modern technology (Marco et al. 2003). A variety of IVET strategies, including the differential fluorescence induction (DFI) of GFP in cells, host-specific complementation of nutritional auxotrophies, and expression of selectable antibiotic resistance genes, remained the most comprehensive approaches to identify bacterial genes induced in a host- or habit-specific environment (Heithoff et al. 1997; Mahan et al. 1993, 1995; Rainey 1999; Valdivia and Falkow 1996, 1997; Wang et al. 1996). Recently, a novel IVET screening strategy termed habitat-inducible rescue of survival (HIRS) was further developed to identify genes of *P. syringae* that were weakly expressed on plants (Marco et al. 2003). Several limiting factors (e.g., the choice of baseline for genes to be considered 'off' in vitro) also have been reported that influenced the type and number of genes identified from this promoter trap strategy. In addition, the level of induction in vivo also was affected by the location, extent, and duration of these environment-inducible genes in IVET assays (Boch et al. 2002; Marco et al. 2003).

E. chrysanthemi 3937 is an opportunistic necrotrophic bacterial pathogen that attacks a wide range of plant species (Chatterjee et al. 2000; Collmer and Keen 1986; Hugouvieux-Cotte-Pattat et al. 1996; Perombelon and Kelman 1980). Until now, limited information was available on gene expression profiles of *E. chrysanthemi* 3937 during host–microbe interactions (Aguilar et al. 2002; Okinaka et al. 2002). To discover plant upregulated genes of *E. chrysanthemi* 3937 in a genome-wide level, we combined the genome analysis database ASAP with a GFP-based IVET leaf array to do a mass screening of bacterial genes that are specifically upregulated in spinach. Compared with the plant-upregulated genes of *E. chrysanthemi* identified from the previous microarray analysis (Okinaka et al. 2002), only a few identical genes (e.g., *yfe* and *hmuU*) were identified in our IVET assay. However, many different genes with similar functions were identified in microarray and IVET studies. The major plant-upregulated genes with similar functions identified in both assays include chemotaxis genes; genes homologous to PTS; genes related to xenobiotic resistance; putative oxidoreductases; genes involved in transposi-

Table 2. Plant-upregulated genes of *Erwinia chrysanthemi* 3937 identified from a green fluorescent protein-based in vivo expression technology leaf array assay using spinach as a host plant

Clone ID	ASAP feature ID	Homologous gene (organism)	Product, functions
PUP-1	19012	*dspE* (*Erwinia amylovora*)	Type III secretion system effector
PUP-2	19593	*hrpA* (*E. carotovora*)	Type III protein secretion system pilus subunit
	19592	*hrpB* (*E. carotovora*)	HrpB, type III protein secretion system
PUP-3	18752	*cheR* (*Yersenia pestis*)	Chemotaxis protein methyltransferase
PUP-4	18585	*trg* (*Escherichia coli*)	Methyl-accepting chemotaxis protein III, ribose sensor receptor
PUP-5	14870	*rhiT* (*E. chrysanthemi*)	Rhamnogalacturonide transporter
PUP-6	20728	*outF* (*E. carotovora*)	Type II secretory pathway component
	20727	*outG* (*E. carotovora*)	Type II secretory pathway component
PUP-7	20503	*nagE* (*Y. pestis*)	PTS system, N-acetylglucosamine-specific IIABC
PUP-8	15122	*yfeA* (*Y. pestis*)	Iron (chelated) ABC transporter, periplasmic protein
PUP-9	19507	*hmuS* (*Y. pestis*)	Hemin degrading protein HmuS
	19508	*hmuT* (*Y. pestis*)	ABC transporter, periplasmic hemin-binding protein
	19509	*hmuU* (*Y. pestis*)	Hemin ABC transporter, permease
PUP-10	16084	*indA* (*E. chrysanthemi*)	Indigoidine biosynthesis protein IndA
PUP-11	17182	*ppK* (*Escherichia coli K12*)	Polyphosphate kinase
PUP-12	14816	*msrA* (*E. chrysanthemi*)	Peptide methionine sulfoxide reductase
PUP-13	16562	*iaaM* (*Pseudomonas agglomerans*)	Tryptophan 2-monooxygenase
PUP-14	16562	*iaaM* (*P. agglomerans*)	Tryptophan 2-monooxygenase
PUP-15	17720	*ppc* (*Escherichia coli*)	Phosphoenolpyruvate carboxylase
PUP-16	18371	*upp* (*Escherichia coli K12*)	Uracil phosphoribosyltransferase
PUP-17	17543	*phoB* (*Escherichia coli*)	Two-component system Pi regulon response regulator
PUP-18	19992	*relB* (*Vibrio cholerae*)	RelB protein
PUP-19	17715	*metR* (*Y. pestis*)	LysR-family transcriptional regulator
PUP-20	15482	*crp* (*E. chrysanthemi*)	CRP regulator
PUP-21	19359	*eutR* (*Salmonella typhimurium*)	Ethanolamine operon regulator
PUP-22	16098	*xylR* (*Escherichia coli K12*)	Xylose operon regulator, AraC-family
PUP-23	20033	*srpS* (*P. putida*)	Transcriptional regulator, Crp family
PUP-24	18849	*atu0245* (*Agrobacterium tumefaciens*)	Transcriptional regulator, Cro/CI family
PUP-25	20364	*yhjH* (*S. typhimurium*)	Diguanylate cyclase/phosphodiesterase domain 3
PUP-26	16993	*pheA* (*P. agglomerans*)	Chorismate mutase-P/prephenate dehydratase
PUP-27	17052	*rimJ* (*Escherichia coli K12*)	Acetyltransferase
PUP-28	18478	*PA4512* (*P. aeruginosa*)	Dioxygenase
PUP-29	16414	*y2956* (*Y. pestis*)	Virulence protein
PUP-30	16412	*yicJ* (*Escherichia coli*)	Sodium galactoside symporter
PUP-31	15477	*YPO0181* (*Y. pestis*)	ABC transport protein
PUP-32	19554	*STY0495* (*S. enterica*)	Exported protein

(*Continued on next page*)

tion, recombination, or DNA repair; putative iron acquisition genes; and other transporters. Using the microarray assay, RNA expression profiles were determined at the instant of sampling time. On the other hand, using a GFP-based reporter, an accumulation of the upregulated signals from bacterial inoculation until harvest was recognized. Different host plants were used in our microarray and IVET assays. These might explain why, in many cases, different plant-upregulated bacterial genes with similar functions were identified in microarray and IVET assays.

With the advantage of the stability of the long-life GFP, host-upregulated genes of *E. chrysanthemi* 3937 during the 18-h infection period in spinach were recognized and divided into different functional categories. Our study indicated that 70% of these plant-upregulated genes in *E chrysanthemi* 3937 could be assigned to functional categories. During disease development, a similar ratio of genes involved in regulation (11%), transport (11%), and cell process (11%) were upregulated in *E. chrysanthemi* 3937. It is possible that the induction of transposase genes (2%) in the plant host may have occurred from the stress in the plant environment (Ilves et al. 2001; Okinaka et al. 2002).

Several genes were identified with probable roles in stress response to reactive oxygen species. These included peptide methionine sulfoxide reductase (*msrA*) and the *indA*. IVET work done in *Streptococcus gordonii* also identified the *msrA* gene induced in endocarditis of the New Zealand white rabbit (Kilic et al. 1999). MsrA can repair oxidatively damaged proteins and is required for full virulence of *E. chrysanthemi* (Hassoouni et al. 1999). IndA is involved in biosynthesis of the blue pigment indigoidine and protects the bacteria against the reactive oxygen species generated during the plant defense response (Reverchon et al. 2002). This might indicate that active oxygen species were produced from the host plant to defend against the invading pathogen and the pathogens make use of different products to protect themselves from the oxidative stresses.

Six transcriptional factors (PhoB, MetR, CRP, EutR, XylR, and SrpS) and one unknown regulator (ASAP ID 18849) were found to be upregulated in spinach in this study. PhoB is a component of a two-component signal transduction pathway. MetR, CRP, EutR, XylR, SrpS, and the unknown regulator contained a helix-turn-helix (HTH) motif either at the N-terminus (MetR, SrpS, and unknown regulator) or at the C-terminus (CRP, EutR, and XylR). PUP-25 contained an EAL domain (pfam00563), which is a good candidate for a diguanylate phosphodiesterase function. In *E. chrysanthemi*, several transcriptional factors related to pathogenicity have been investigated intensely by other researchers. These include the positive regulators CRP, Pir, and ExpR, and negative regulators, such as KdgR, PecS, and PecT (Chatterjee et al. 2000; Franza et al. 1999; Hugouvieux-Cotte-Pattat et al. 1996; Nassar and Reverchon 2002; Nasser et al. 1994, 1998). The recruitment of many regulators may indicate that successful pathogenicity requires complex gene regulation and expression for rapid adaptation to the complicated and di-

Table 2. (*Continued from previous page*)

Clone ID	ASAP feature ID	Homologous gene (organism)	Product, functions
PUP-33	15731	*YPO0661* (*Y. pestis*)	Exported protein
PUP-34	19297	*b1329* (*Escherichia coli K12*)	ABC transporter, periplasmic murein peptide-binding
PUP-35	19750	*glpM* (*Y. pestis*)	Membrane protein
PUP-36	19662	*YPO2922* (*Y. pestis*)	Membrane protein
PUP-37	20770	*RS02521* (*Ralstonia solanacearum*)	Membrane protein
PUP-38	19164	*yhhA* (*S. typhimurium*)	Outer membrane protein
PUP-39	15150	*yaaA* (*S. typhimurium*)	Cytoplasmic protein
PUP-40	16622	*STM4501* (*S. typhimurium*)	Cytoplasmic protein
PUP-41	16622	*STM4501* (*S. typhimurium*)	Cytoplasmic protein
PUP-42	20093	*Cj0014c* (*Campylobacter jejuni*)	Integral membrane protein
PUP-43	15890	*XCC4050* (*Xanthomonas campestris*)	Ankyrin-related protein
PUP-44	17185	*STY0862* (*S. enterica*)	Zinc-finger containing protein
PUP-45	17186	*ynaF* (*S. typhimurium*)	Universal stress protein
PUP-46	17186	*ynaF* (*S. typhimurium*)	Universal stress protein
PUP-47	18343	*crcB* (*Escherichia coli K12*)	Chromosome condensation protein
PUP-48	18876	*XAC3843* (*Xanthomonas axonopodis*)	Unknown protein
PUP-49	16049	Unknown protein	Unknown protein
PUP-50	20543	*yqgA* (*Shigella flexneri*)	Unknown protein
PUP-51	20034	*STY2446* (*S. enterica*)	Unknown protein
PUP-52	17269	*vgrG* (*Y. pestis*)	VgrG-like protein
PUP-53	17269	*vgrG* (*Y. pestis*)	VgrG-like protein
PUP-54	20121	*tnpA* (*E. chrysanthemi*)	Transposase
	20118	*ogl* (*E .chrysanthemi*)	Oligogalacturonate lysase
PUP-55	20619	*ansA* (*Y. pestis*)	Cytoplasmic L-asparaginase I
	19584	*pncA* (*Y. pestis*)	Pyrazinamidase/nicotinamidase
PUP-56	14828	*bacA* (*Y. pestis*)	Undecaprenol kinase, bacitracin resistance protein
	14827	*folB* (*Y. pestis*)	Dihydroneopterin aldolase
PUP-57	17163	*ydfP* (*Bacillus subtilis*)	Unknown protein
	17162	*cysS* (*Y. pestis*)	Cysteinyl-tRNA synthetase
PUP-58	17061	*yecM* (*S. typhimurium*)	Unknown protein
	17062	*cutC* (*Y. pestis*)	Copper homeostasis protein
PUP-59	16275	*pstB* (*Escherichia coli*)	High-affinity Pi ABC transporter, ATP binding protein
	16274	*phoU* (*Escherichia coli*)	Phosphate transport system regulator
	16270	*YPO4111* (*Y. pestis*)	Periplasmic solute-binding protein
PUP-60	20706	*phnC* (*Y. pestis*)	Pi uptake ABC transporter, ATP-binding protein
	20707	*phnD* (*Y. pestis*)	Pi uptake ABC transporter, periplasmic protein
PUP-61	18644	*XCC2096* (*X. campestris*)	Unknown protein

verse plant host environment (Buell et al. 2003). Unraveling the secrets of the temporal and spatial expression pattern in *E. chrysanthemi* 3937 regulators may help us understand its adaptation and fitness in host environments.

Iron is in short supply in numerous environments, and many bacteria secrete siderophores specifically to chelate iron. In our studies, various putative iron-uptake genes, including *hmuSTU* and *yfeA* homologues, were recognized as plant upregulated. Similarly, many genes related to iron metabolism were found to be induced with the IVET approach in other bacterial pathogens. These include *entF* and *fhuA* in *S. typhimurium*, *fur* and *fptA* in *P. aeruginosa*, and *fepA* in *Klebsiella pneumoniae* (Heithoff et al. 1997; Lai et al. 2001; Wang et al. 1996). In *E. chrysanthemi*, two structurally unrelated siderophores, chrysobactin and achromobactin, were identified. Chrysobactin is required for the bacterium to cause systemic infection in African violet. Plant hosts inoculated with an *E. chrysanthemi* achromobactin mutant exhibit delayed symptoms (Expert 1999; Franza et al. 1999).

Two redundant transport systems, TogMNAB and TogT, have been identified in *E. chrysanthemi* 3937 and are able to independently mediate the transport of oligogalacturonides through the inner membrane of this bacterium. Inactivation of both systems is necessary to give a clear phenotype of transportation deficiency (Hugouvieux-Cotte-Pattat and Reverchon 2001; Hugouvieux-Cotte-Pattat et al. 2001). There is strong evidence that the newly identified *rhiT* plays a role in the uptake of rhamnogalacturonides (Hugouvieux-Cotte-Pattat 2004). Rhamnogalacturonides are generated by the extracellular enzyme RhiE, a 62-kDa protein that has rhamnogalacturonate lyase activity on rhamnogalacturonan I (RG-I). The reduced virulence of the *rhiE* mutant indicates that degradation of the RG-I region of pectin is important for full virulence of *E. chrysanthemi* (Laatu and Condemine 2003). In this study, a disruption of *rhiT* in *E. chrysanthemi* 3937 did not significantly alter local maceration, but systemic invasion capability was compromised. The mechanism of reduced systemic invasion ability of the *rhiT* mutant in the host plant is unclear. One possibility is that a deletion of the *rhiT* gene in *E. chrysanthemi* 3937 could disrupt the production of other exoenzymes and pathogenicity factors regulated by degraded pectin and pectate intermediates (Toth et al. 2003). The precise production of these virulence factors during the infection process might be required for a systemic invasion of *E. chrysanthemi* 3937 in host plants. In any event, the *rhiT* mutant further reveals the importance of pectin utilization for *E. chrysanthemi* pathogenicity. Similarly, *eutR*::Tn*5* mutants of *E. chrysanthemi* 3937 had a reduced capacity to invade systemically. Gathering more information from further local and systemic infection assays may perhaps help us explain the mechanism of delayed systemic infection.

Several plant-upregulated genes of *E. chrysanthemi* 3937 related to phosphate metabolism, transportation, and regulation were identified through the IVET assay. They include *ppK*,

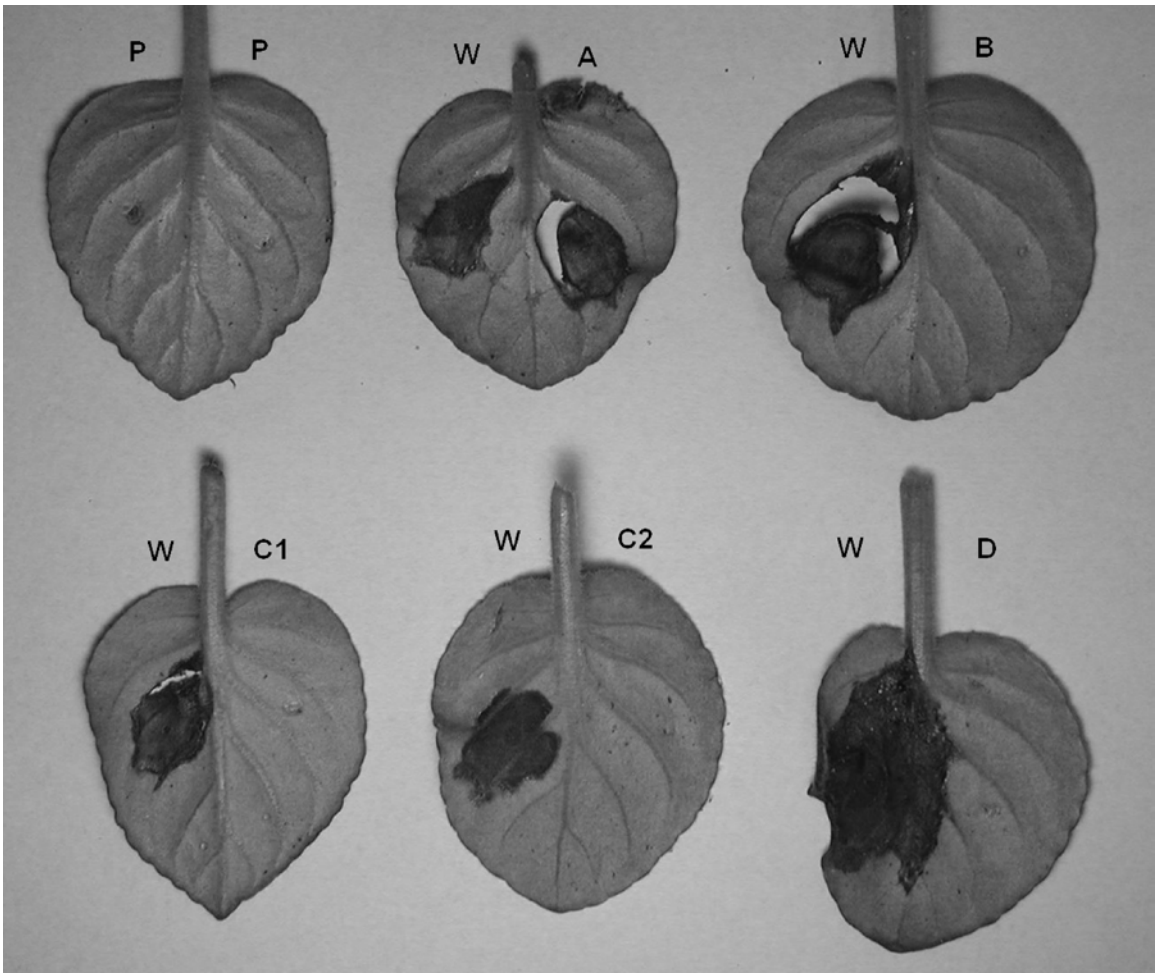

Fig. 1. Local maceration lesions caused by wild-type *Erwinia chrysanthemi* 3937 (W) and mutants *pup2D*::Tn*5* (A), *hrpD*::Tn*5* (B), *hrcJ*::Tn*5* (C1 and C2), and *hrpB*::Tn*5* (D) on leaves of African violet cv. Katja. Bacterial cells were inoculated in the middle of each half side of the same leaf. Phosphate buffer (pH 7.4, 50 mM) were used to suspend the bacterial cells and a volume of 0.05 ml of bacterial suspension with a bacterial concentration of 10^6 CFU/ml was used. P indicates leaves infiltrated with phosphate buffer alone. Both C1 and C2 are *hrcJ* mutants with Tn*5* transposon insertion at different locations of *hrcJ* gene.

phoB, *pstB*, *phnC*, *phnD*, and a potential Pi ABC transporter periplasmic substrate-binding component homologue. Inorganic polyphosphate (Poly P) is a chain of many hundreds of phosphates (Pi) linked by high-energy phosphoanhydride bonds and widespread in all life forms. PPK is a bifunctional polyphosphate kinase that synthesizes polyphosphates from ATP or converts poly P and ADP to ATP. There are several potential functions for cellular Poly P, including a phosphate and energy reservoir, a chelator of metal ions, a buffer against alkali, and a channel for DNA entry (Kim et al. 2002; Kornberg et al. 1999; Shiba et al. 2000). Some evidence has been gathered for the role of *ppK* in virulence. For example, inorganic polyphosphate was required for motility of bacterial pathogens, and *ppK* of *P. aeruginosa* is responsible for biofilm development, quorum sensing, and virulence (Rashid and Kornberg 2000; Rashid et al 2000a and b). Von Kruger and associates (1999) verified that a *Vibrio cholerae phoB* mutant was less able to colonize rabbit intestines than the wild-type bacterium. It will be interesting to further investigate whether or not the *ppK* and *phoB* of *E. chrysanthemi* 3937 are important for bacterial response to Pi starvation, virulence gene expression, and survival.

The bacterial PTS catalyzes sugar phosphorylation and dephosphorylation, and regulates a variety of bacterial physiological processes. It plays important roles in chemotaxis, intermediary metabolism, gene transcription, and virulence (Saier and Reizer 1994). In our IVET assay, a *nagE* homologue in *E. chrysanthemi* 3937 was upregulated in the plant host. NagE is a component of an N-acetylglucosamine-specific IIABC PTS system, containing a PTS-EIIC conserved domain at the N-terminus and a PTS-EIIB conserved domain at the C-terminus.

Bacteria use chemotaxis to respond to microenvironments by swimming toward or away from stimulants to optimize their growth and survival. Although a lot of work has been carried out on the role of motility as virulence factor for bacteria (Gosink et al. 2002; Larsen and Boesen 2001; O'Toole et al. 1996), little has been done on *E. chrysanthemi*. This study identified the *cheR* and *trg* homologues in *E. chrysanthemi* 3937 as upregulated in the plant host. An additional investigation about the interactions among chemotaxis, PTS, and Pi systems identified in the IVET assay may lead to an understanding of the regulatory mechanisms involved in the bacterial infection process. In addition, the upregulation of *nagE* (N-acetylglucosamine-specific PTS system component), *b1329*

Table 3. Local lesion maceration by wild-type *Erwinia chrysanthemi* 3937 and mutants on African violet cv. Katja

Mutant[a]	Potential function of mutated gene	ASAP ID[b]	PI[c]
ECH16412::Tn*5*	Putative sodium galactoside symporter	16412	0.708 ± 0.135
rhiT::Tn*5*	Rhamnogalacturonide transporter	14870	1.821 ± 0.830
uraA::Tn*5*	Uracil transporter	18369	1.063 ± 0.344
upp::Tn*5*	Uracil phosphoribosyl transferase	18371	0.807 ± 0.165
purM::Tn*5*	Phosphoribosylaminoimidazole synthetase	18373	0.047 ± 0.061
hrpB::Tn*5*	Unknown, possible secretion	19592	0.019 ± 0.032
hrcJ::Tn*5*	Lipoprotein family protein	19590	0
hrpD::Tn*5*	Unknown, possible secretion	19588	0.016 ± 0.014
pup2D::Tn*5*	Putative Membrane-bound lytic murein transglycosylase	19586	1.235 ± 0.222
eutR::Tn*5*	Putative ethanolamine operon regulatory protein	19359	0.794 ± 0.093

[a] Except for the *purM* mutant, all mutant strains grew as well as the wild-type on M9 glucose minimal medium.

[b] Identification numbers: 16412, 14870, 18371, 19592, and 19359 are plant-upregulated genes found in IVET screening; 18369 and 18373 are genes flanking 18371; 19590, 19588, and 19586 are genes flanking 19592.

[c] Pathogenicity index (PI) = Σ(lesion size of leaves from the bacterial mutant/lesion size of leaves from the wild-type bacterium)/number of leaves used for measurement. PI < 1, the bacterial mutants had reduced virulence compared with the wild-type bacterium; PI = 1, the bacterial mutants were as virulence as the wild-type bacterium; PI > 1, the bacterial mutants had enhanced virulence compared with the wild-type bacterium. Maceration area was calculated with American Phytopathological Society Assess software (St. Paul, MN, U.S.A.). Three replicate plants with a total of 12 leaves were inoculated for each of the wild-type bacterium and its mutants.

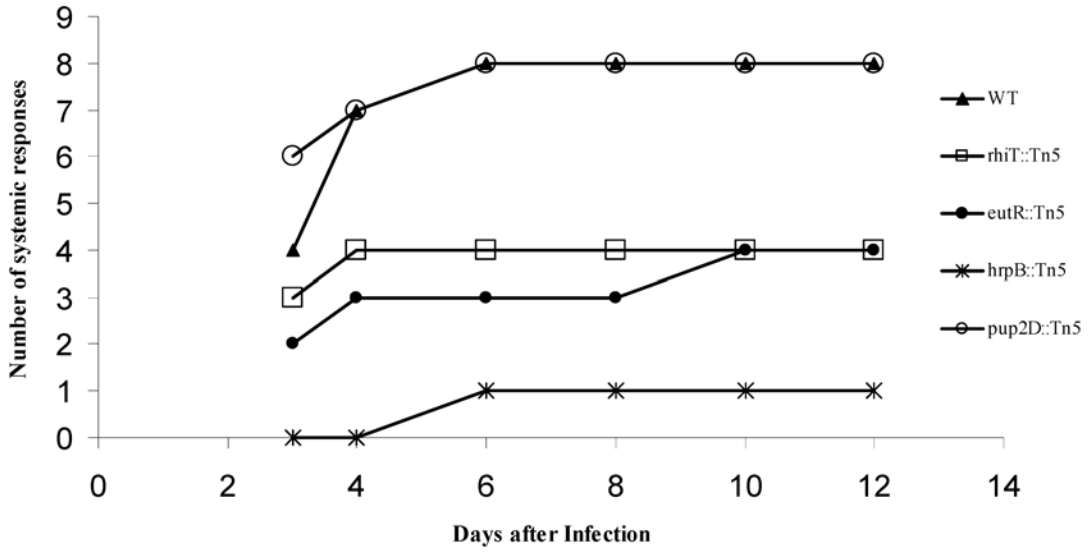

Fig. 2. Development of symptoms induced by *Erwinia chrysanthemi* 3937 wild type and *rhiT*::Tn*5*, *eutR*::Tn*5*, *hrpB*::Tn*5*, and *pup2D*::Tn*5* mutant strains on African violet cv. Katja plants. A systemic invasion assay was used as described by Nachin and associates (2001) with minor modification. For each strain, 10 plants (one leaf per plant) were inoculated. Response was considered as systemic when at least one leaf and its petiole were macerated.

(ABC transporter, periplasmic murein peptide-binding protein), and *bacA* (undecaprenol kinase, bacitracin resistance protein) all may be related to the increased need for cell wall growth during bacteria proliferate within the plant environment.

Various genes related to purine and pyrimidine biosynthesis have been reported in IVET and signature-tagged mutagenesis (STM) work. These include *purCELK* in *Streptococcus* spp.; *purDHK*, *purKLFC*, and *carBA* in *V. cholerae*; and *purHLD* in *P. aeruginosa*. The interruption of these genes may reduce the virulence of these pathogens in their hosts (Chiang and Mekalanos 1998; Lehoux et al. 2002; Merrell et al. 2002; Polissi et al. 1998). Although the *purM* homolog mutant (*purM*::Tn*5*) of *E. chrysanthemi* 3937 showed reduced virulence on African violet, it was unable to grow on M9 minimum medium. The cause of reduction in virulence of this mutant may be due to its auxotrophic phenotype. Other genes found in this IVET study were the genes involved in xenobiotic resistance, as well as several transposase genes that also were reported by other groups using IVET or STM approaches (Boch et al. 2002; Coulter et al. 1998; Kilic et al. 1999; Merrell et al. 2002; Wang et al. 1996; Wu et al. 2002).

Although the type III secretion system has been identified in *E. chrysanthemi*, relatively little is known about its role in pathogenicity (Bauer et al. 1994, 1995; Lopez-Solanilla et al. 2001; Yang et al. 2002). The mutants of *hrpB*, *hrcJ*, and *hrpD* homologues from the *hrpA* operon of *E. chrysanthemi* 3937 had reduced local leaf maceration and reduced systemic invasion abilities, which further confirmed our previous result that *hrp* genes in *E. chrysanthemi* 3937 are crucial for full virulence (Yang et al. 2002).

The *rhiT* and *eutR* genes of *E. chrysanthemi* 3937 identified in this study can be classified as class III virulence genes. However, a significant proportion of bacterial genes upregulated in plant hosts in this study are not necessarily for its pathogenicity. *E. chrysanthemi* is a bacterial pathogen that can infect a wide range of plant species. In this study, we identified a broad spectrum of genes in *E. chrysanthemi* 3937 that were upregulated in spinach. With the completion of *E. chrysanthemi* 3937 genome sequencing, it will be interesting to further compare arrays of genes in the bacterium that are regulated in different plant hosts and study how the bacterium coordinates gene expression to adapt to different host environments.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *E. chrysanthemi* 3937 and mutant strains were stored at –80°C in 15% glycerol and grown on LB agar. Antibiotics used were kanamycin at 50 μg/ml and ampicillin at 100 μg/ml. Primers used for polymerase chain reaction (PCR) in this report also are listed in Table 1.

pPROBE-AT:P*pelD*, pPROBE-AT:P*hrpG*, and pPROBE-AT:P*hrpN* promoter vector construction.

DNA fragments of *pelD*, *hrpG*, and *hrpN* promoter regions were PCR amplified from *E. chrysanthemi* 3937 chromosomal DNA. The amplified DNA fragments were gel purified with QIAquick gel extraction kit (Qiagen, Valencia, CA, U.S.A.) and ligated into the pGEM-T Easy vector system containing 3′T overhangs at the insertion side (Promega Corp., Madison, WI, U.S.A.). The plasmid DNA was digested with *Eco*RI to release the *pelD*, *hrpG*, and *hrpN* promoter fragments which were ligated into the promoter-probe vector pPORBE-AT at the *Eco*RI site. The orientation of the inserted DNA fragments in pPROBE-AT was determined by PCR. Plasmids containing DNA inserts with the correct (promoter transcribes toward GFP promoter-probe cassettes) and the opposite orientations were selected. These plasmids were designated as pPROBE-AT::P*pelD*, pPROBE-AT::P*hrpG*, pPROBE-AT::P*hrpN* (promoter with the transcription orientation toward the promoterless GFP reporter of pPROBE-AT), pPROBE-AT::P*pelD*op, pPROBE-AT::P*hrpG*op, and pPROBE-AT::P*hrpN*op (promoter with the transcription orientation opposite to promoterless GFP reporter). Seven different plasmids, pPROBE-AT::P*pelD*, pPROBE-AT::P*hrpG*, pPROBE-AT::P*hrpN*, pPROBE-AT::P*pelD*op, pPROBE-AT::P*hrpG*op, pPROBE-AT::P*hrpN*op, and the pPROBE-AT vector alone, were electroporated individually into wild-type *E. chrysanthemi* 3937. Plasmid-containing bacteria were inoculated into spinach leaves and LB media. The GFP protein expression from bacteria was observed using an Olympus fluorescent microscope.

GFP-based IVET leaf array screening strategy.

A library of 1.5-kb fragments of *Sau3A*1 partially digested genomic DNA of wild-type *E. chrysanthemi* 3937 was cloned by ligation into the *Bam*HI site of the promoter-probe vector, pPROBE-AT. The 1.5-kb chromosomal fragments were size fractionated through agarose gel eletrophoresis and further purified through QIAquick gel extraction kit. The pPROBE-AT plasmid contained a broad host-range replicon from pBBR1 and a promoterless GFP reporter. Random library clones constructed in pPROBE-AT were electroporated into wild-type *E. chrysanthemi* 3937 as described (Yang et al. 2002). The plasmid-containing bacteria were grown on LB agar at 28°C overnight and transferred into microtiter plates containing 3-mm discs cut from market-purchased spinach leaves. Microtiter plates containing plant leaves and bacteria were vacuum infiltrated in a closed chamber to introduce bacteria into the leaf discs (with a vacuum pressure of 500 mmHg for 3 min, repeated six times). All leaf discs were incubated at 28°C for 18 h with moderate shaking. As controls, we used bacteria carrying the pPROBE-AT vector alone and plasmids pPROBE-AT::P*pelD*, pPROBE-AT::P*hrpG*, and pPROBE-AT::P*hrpN* that contained known *E. chrysanthemi* plant-inducible promoters of *hrpG*, *pelD*, and *hrpN*. After incubation, the leaf discs were mechanically macerated to release bacterial cells. The cell suspension was spotted on glass plates by using a hand arrayer and observed under a fluorescent microscope to monitor relative GFP fluorescence. Fluorescent bacteria were selected and rescreened on LB liquid medium and in spinach leaf discs. Bacterial clones leading to fluorescence of similar intensity between host leaves and LB broth were assumed to contain constitutive promoters and were discarded. Bacteria carrying plant upregulated promoters (no or lower fluorescence on LB medium compared with host leaf) were confirmed by rescreening on leaves and LB media. The consistent clones were end sequenced to identify the cloned genes or promoter regions.

Sequencing and annotating candidate clones.

Initially, sequencing primers were designed for primer walking to complete the full length of the insert DNA fragments in pPROBE-AT if end sequencing did not give the full length of the insert DNA fragments. The vector sequences were trimmed and remaining DNA sequences were assembled in Vector NTI (InforMax Inc., Bethesda, MD, U.S.A.). The ORF prediction and homologue search of ORFs were analyzed by Vector NTI through NCBI Blast searches. After the preliminary *E. chrysanthemi* 3937 genome sequence became available, the candidate clones were end sequenced from both ends and compared in ASAP to obtain the full-length sequence of insert DNA. The functional groups of plant-upregulated genes from our IVET assay were categorized accordingly (Glasner et al. 2003; Serres and Riley 2001). Several other bioinformatics

tools were used to analyze their conserved motifs and putative functions, such as NIH NCBI Conserved Domain Database and Search Service (CDD), Kyoto Encyclopedia of Genes and Genomes (KEGG), and *Escherichia coli* genome and proteome databases (GenProtEC).

Exploring the potential functions of plant-upregulated genes by Tn*5* mutagenesis and virulence assays.

Initially, a pooled library screening strategy was used to identify *Erwinia chrysanthemi* 3937 library clones containing target genes for mutation. Specific PCR primers were designed for a 7- to 8-kb DNA insert library pool (48 library clones were pooled as one screening unit) of *E. chrysanthemi* 3937. Once an expected PCR DNA fragment was observed in *E. chrysanthemi* library pools, 48 insert library clones were amplified individually to obtain the library clone containing a target gene. Selected ORFs on plant-upregulated plasmid clones were mutagenized with Tn*5* transposon using an EZ::TN <KAN-2> insertion kit with the procedures suggested by the manufacturer (Epicentre Technologies, Madison, WI, U.S.A.). Tn*5* insertions were physically mapped and precisely located by sequencing with transposon primers IR-F or IR-R. The plasmid constructs then were electroporated into *E. chrysanthemi* 3937 and plasmid curing and marker exchange were accomplished by growth of kanamycin-resistant transformants in low-phosphate medium broth (Ried and Collmer 1987; Yang et al. 2002). Mutations in the resultant kanamycin-resistant and ampicillin-sensitive colonies then were confirmed by PCR, using transposon and target gene-specific primers.

Subsequently, PCR primers were designed for plant-upregulated clones or genes based on the available *E. chrysanthemi* 3937 genome data. DNA fragments were amplified using a proofreading DNA polymerase (FailSafe PCR system; Epicentre), cloned into pGEM-Teasy vector, and mutated with Tn*5*, after which marker exchange mutagenesis was performed as described above.

Two different virulence assays, local leaf maceration and systemic invasion, were conducted on African violet cv. Katja leaves. In the local maceration assay, a volume of 0.05 ml with a bacterial concentration of 10^6 CFU/ml of wild-type *E. chrysanthemi* 3937 and its mutants was inoculated in the middle of each half side of the same leaf. Phosphate buffer (pH 7.4, 50 mM) was used to suspend the bacterial cells. The inoculated plants were placed in a growth chamber under the conditions described previously (Yang et al. 2002). Three replicate plants with a total of 12 leaves were inoculated for each of the wild-type bacterium and its mutants. The maceration area of plant leaves caused by the bacterial strains was precisely measured with an image analysis software, ASSESS (The American Phytopathological Society, St. Paul, MN, U.S.A.). The PI for each bacterial mutant was calculated according to the equation PI = Σ(lesion size of leaves from the bacterial mutant/lesion size of leaves from the wild-type bacterium)/number of leaves used for measurement.

In the systemic invasion assay, the pathogenicity of *E. chrysanthemi* 3937 was evaluated as described by Nachin and associates (2001) with minor modification. A volume of 0.05 ml of bacterial suspension with an optical density at 600 nm of 0.3 was inoculated into the front edge of the African violet cv. Katja leaf. Inoculated plants were kept in a growth chamber with regular water misting (5 min in every 4 h) to provide humid conditions.

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