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Identification of *hrpL* up-regulated genes of *Dickeya dadantii*

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Abstract *Dickeya dadantii* (*Erwinia chrysanthemi*) is a Gram-negative plant pathogen that invades a wide range of plant species to cause diseases. Hypersensitive response and pathogenicity genes (*hrp*) are important virulence factors in *D. dadantii*. However, few *hrpL* up-regulated genes of *D. dadantii* have been reported. In this study, a green fluorescence protein (GFP)-based *Escherichia coli* promoter-probe system was used to identify *hrpL* up-regulated genes in *D. dadantii* 3937. From approximate 20,000 library clones screened, *hrpA, hrpK, dspE*, *yijC* (a DNA-binding transcriptional regulatory protein) and *yecF* (unknown function) were identified to be up-regulated by *hrpL* in *D. dadantii* 3937. Expression pattern fluorescence of five genes was observed in the wild-type strain and a *hrpL* deletion mutant strain of *D. dadantii* 3937 in *hrp*-inducing minimal medium. Mutants with *hrpA, hrpK, dspE, yijC*, and *yecF* genes mutated, respectively, were confirmed, and most of the mutants showed virulence reduction when infecting the plant host African violet (*Saintpaulia ionantha*).

Keywords Effector proteins · *hrp* · Type III secretion system · Virulence factors · *yecF* · *yijC*

Abbreviations

ECF	extra cytoplasmic function
Ech3937-*D. dadantii* 3937	(*Erwinia chrysanthemi* 3937)
EEL	exchangeable effector locus
GFP	green fluorescence protein
Hop	*hrp* outer protein
HR	hypersensitive responses
hrp	hypersensitive response and pathogenicity
IVET	in vivo expression technology
MM	*hrp* inducing minimal medium
ORF	open reading frame
PAI	pathogenicity island
P*Ech3937*	an Ech3937 pPROBE-AT library
T3SS	Type III secretion system
RT-PCR	reverse transcription polymerease chain reaction

Introduction

Dickeya dadantii (syn. *Erwinia chrysanthemi*, *Pectobacterium chrysanthemi*) (Samson et al. 2005) has a wide host range and is an opportunistic plant pathogen that survives in soil, water, and the surface of plants with no pathogenic phase (Chatterjee et al. 2000; Stanghellini 1982). Once in the pathogenic

This article is in memory of Professor Noel T. Keen who passed away on April 18, 2002.

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phase, it causes diverse disease symptoms including economically important soft-rot, wilt and blight diseases. Several virulence mechanisms in *D. dadantii*, such as a siderophore-dependent iron uptake system, the type II secretion system for secreting pectate lyase and cellulose, and the type III secretion system (T3SS) (Barras et al. 1994; Bauer et al. 1994; Expert 1999; Franza and Expert 1991) have been studied.

Previous studies showed that T3SS was required for elicitation of defencive hypersensitive responses (HR) on non-host plants and pathogenicity on host plants, thus called *hrp* (*h*ypersensitive *r*esponses and *p*athogenicity) (Bonas 1994; Galan and Collmer 1999). The *hrp* genes are clustered and conserved in Gram-negative plant pathogenic bacteria, such as *Pseudomonas syringae*, *E. amylovora* and *Xanthomonas campestris* (Alfano et al. 2000; Bogdanove et al. 1998; He 1998). *hrp* genes encode proteins with roles in gene regulation, assembly of the *hrp* pili, and secretion of effector proteins into plant cells (Collmer et al. 2002). It is predicted that the functions of the effector proteins are to modulate the physiology of the host cell for bacterial survival (Jin et al. 2003).

In *E. amylovora*, the two-component regulatory system HrpX/HrpY senses the environmental signals. Cooperating with HrpS, HrpX/HrpY up-regulates HrpL, a member of the extra cytoplasmic function (ECF) sigma factor family (Chatterjee et al. 2002; Wei et al. 2000). HrpL recognises consensus promoter sequences called Hrp box, and activates expression of genes encoding the T3SS and its secreted effector proteins (Gaudriault et al. 1997; Wei et al. 2000).

Although several *D. dadantii* 3937 (Ech3937) mutants, including the *hrcC* and *hrpG* homologues from the *hrpC* operon, and *hrpB*, *hrcJ*, and a *hrpD* homologue from the *hrpA* operon, showed reduction of growth in African violet plants (Yang et al. 2002, 2004), few *hrpL* regulated genes of *D. dadantii* have been reported to date. In this study, a green fluorescence protein (GFP)-based *Escherichia coli* promoter-probe system was used to screen genes up-regulated by *hrpL* of Ech3937. The *hrpL* up-regulated genes were further characterised in *Escherichia coli* carrying pCPP30421 (*hrpL* deficient *hrp* cluster) and in Ech131 (a *hrpL* deletion mutant of Ech3937), respectively. Finally, the role of *hrpL* up-regulated genes in Ech3937 virulence is discussed.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Wild-type Ech3937 and its mutant strains were stored at −80°C in 15% glycerol and grown at 28°C on LB agar. *Hrp*-induced minimal media (MM) were prepared as descried previously (Huynh et al. 1989). Media were supplemented with antibiotics, when indicated, at the following concentrations (μg ml^{-1}): kanamycin, 50; ampicillin, 100; tetracycline, 15; spectinomycin, 50; streptomycin, 50.

The construction of plasmids

Plasmid pCPP3042 containing the entire *D. dadantii* EC16 *hrp* cluster (Ham et al. 1998) was mutagenised, with Tn5 transposon using the EZ::TN™ <KAN-2> insertion kit per manufacturer's instructions (Epicentre Technologies, MI). The plasmid with Tn5 insertion in *hrpL* open reading frame (ORF) of *hrp* cluster was named pCPP30421 (Table 1). The precise location of the Tn5 insertion in *hrpL* was confirmed by DNA sequencing using transposon primers Kan-1 FP-1 or Kan-1 RP-1 (Table 2).

The *hrpL* gene (the promoter and ORF) of Ech3937 was amplified by PCR with primers HrpL For/Rev (Table 2) and cloned into pGEM-T Easy (Promega, MI) (Table 1). The 0.624 kb PCR fragment released from pGEM-T Easy by *Not*I was ligated into pDSK600 (Keen et al. 1988) and pRK415 (Keen et al. 1988) digested with *Not*I for constructing pDSK6001 and pRK4151 (Table 1), respectively.

Identification of hrpL up-regulated genes

Genomic DNA from Ech3937 was partially digested with *Sau3*A1. One to 2 kilobase fragments were cloned into the *Bam*H1 site of pPROBE-AT (Miller et al. 2000), which contained a broad-host-range replicon from pBBR1 and a promoterless GFP reporter gene. Recombinant pPROBE-AT plasmid DNA was electroporated into *E. coli* DH5α carrying pCPP3042, and grown on LB plates with ampicillin (100 μg ml^{-1}) and tetracycline (15 μg ml^{-1}) at 28°C overnight, to construct an Ech3937 pPROBE-AT library (P*Ech3937*). The recombinant clones of P*Ech3937* were identified by blue/white clone

Table 1 Bacterial strains, plasmids and primers used in *D. dadantii* experiments

Strains and plasmids	Characters and primers	Source
Bacterial strains		
Escherichia coli DH5α	DH1 F^-, Φ80 Δ*LacZ*ΔM15, Δ(*lacZYA-argF*), U169	
Ech3937	Wild-type *D. dadantii* 3937	Hugouvieux-Cotte-Pattat, N.
Ech131	The *hrpL* deletion mutant of Ech3937 (Δ*hrpL::Km*)	This work
Ech132	The *hrpK* deletion mutant of Ech3937 (Δ*hrpK::Km*)	This work
Ech133	The *dspE* deletion mutant of Ech3937 (Δ*dspE::Km*)	This work
Ech134	The *yecF* deletion mutant of Ech3937 (Δ*yecF::Km*)	This work
Ech135	The *yijC* deletion mutant of Ech3937 (Δ*yijC::Km*)	This work
Ech136	The *hrpA* deletion mutant of Ech3937 (Δ*hrpA::Km*)	This work
Plasmids		
pPROBE-AT	Ap^R, the promoter-probe vector	Miller et al., (2000)
P*hrpN*	Ap^R, *hrpN* promoter cloned into pPROBE-AT	Yang et al., (2002)
P*hrpG*	Ap^R, *hrpG* promoter cloned into pPROBE-AT	Yang et al., (2002)
pDSK600	Gm^R, the broad-range plasmid	Keen et al., (1988)
pRK415	Tc^R, the broad-range plasmid	Keen et al., (1988)
pDSK6001	Sp^R/Sm^R, *hrpL* of Ech3937cloned in pDSK600	This work
pRK4151	Tc^R, *hrpL* of Ech3937 cloned in pRK415	This work
pCPP3042	Tc^R, the completely *hrp* gene cluster from *D. dadantii* EC16	Ham et al., (1998)
pCPP30421	pCPP3042 with Tn5 insertion at *hrpL* ORF	This work
pGEM-T Easy	Ap^R, the cloning vector	Promega. WI
pGEMT-L	Ap^R, the final PCR fragments containing the left and right sides of *hrpL*	This work
pGEMT-K	Ap^R, the final PCR fragments containing the left and right sides of *hrpK*	This work
pGEMT-E	Ap^R, the final PCR fragments containing the left and right sides of *dspE*	This work
pGEMT-F	Ap^R, the final PCR fragments containing the left and right sides of *yecF*	This work
pGEMT-C	Ap^R, the final PCR fragments containing the left and right sides of *yijC*	This work
pGEMT-A	Ap^R, the final PCR fragments containing the left and right sides of *hrpA*	This work
pGEMT-3	Ap^RKm^R, Kanamycin cassette with *Asc*I site in pGEM-T	This work
pGEMT-LK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-L	This work
pGEMT-KK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-K	This work
pGEMT-EK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-E	This work
pGEMT-FK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-F	This work
pGEMT-CK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-C	This work
pGEMT-AK	Ap^RKm^R, a kanamycin cassette was cloned into *AscI* site of pGEMT-A	This work
pBSK1	Cm^R, *sacB* gene cloned in down-stream of Cm cassette in pBSK(+)	Yang et al., (2002)
pBSK-LK	Cm^RKm^R,the mutagenic fragments from pGEMT-LK cloned into pBSK1	This work
pBSK-KK	Cm^RKm^R,the mutagenic fragments from pGEMT-KK cloned into pBSK1	This work
pBSK-EK	Cm^RKm^R,the mutagenic fragments from pGEMT-EK cloned into pBSK1	This work
pBSK-FK	Cm^RKm^R,the mutagenic fragments from pGEMT-FK cloned into pBSK1	This work
pBSK-CK	Cm^RKm^R,the mutagenic fragments from pGEMT-CK cloned into pBSK1	This work
pBSK-AK	Cm^RKm^R,the mutagenic fragments from pGEMT-AK cloned into pBSK1	This work

selection on the plates. Individual white colonies were transferred into 96-well microtiter plates containing 150 μl MM in each well. The microtiter plates were incubated at 28°C for 18 h. *Escherichia coli* DH5α carrying either pPROBE-AT, pCPP3042, P*hrpN* or P*hrpG*, was used as the negative controls (Table 1). *Escherichia coli* DH5α carrying P*hrpN*/pCPP3042 and P*hrpG*/pCPP3042 was used as the positive controls, which contained the promoters of *hrp* genes that should be regulated by *hrpL* to produce fluorescence.

After incubation, the *E. coli* cells containing pCPP3042 and the recombinant clones of P*Ech3937* in individual wells of microtiter plates were observed under a fluorescence microscope (Carl Zeiss 510 Laser Scanning Confocal Microscope, 40 X, green colour filters, 458 nm) to monitor the relative fluorescence. The fluorescent individual bacterial culture, which

Table 2 Primers used in this study

Primers	Sequences	The expected PCR size (bp)	Source
pPROBE-AT For	5′-ACTGCCAGGAATTGGGGATCGGAAG-3′		This work
pPROBE-AT Rev	5′-AGTTCTTCTCCTTTACTCATA-3′		This work
Kan-1 FP-1	5′ -ACCTACAACAAAGCTCTCATCAACC-3′		Epicentre
Kan-1 RP-1	5′ -GCAATGTAACATCAGAGATTTTGAG-3′		Epicentre
HrpL For	5′-TTATGCATCAACAGCCTGGC-3′	624	This work
HrpL Rev	5′-GGTCCTGTCCTGCCTGAAGC-3′		This work
HrpL-AF1	5′- CGCCAGCAGTCGCAGCTGCA-3′	1,059	This work
HrpL-BR2	5′-ATCCTGCTTTCGGCGCGCCGTCGCGTCTTTCCCGCGCCAG-3′		This work
HrpL-CF3	5′-CGGCGCGCCGAAAGCAGGATTGAATCTGACACGCATCCA-3′	890	This work
HrpL-DR4	5′-TATCAGACGTCGGGTCAGCT-3′		This work
HrpK-AF1	5′-TCAATCAAATGAAAAGGCGG-3′	631	This work
HrpK-BR2	5′-TTCGGCGCGCCGAATCCTTAATTAAGGGGTGTCGATTCCCCTCAGAA-3′		This work
HrpK-CF3	5′-CCTTAATTAAGGATTCGGCGCGCCGAAGTTATCCGTTGCGTTTAACG-3′	597	This work
HrpK-DR4	5′-CTGCCGGTGCGCTGGCTGGC-3′		This work
DspE-AF1	5′-CAGCATATCAAGCAGTTTGG-3′	675	This work
DspE-BR2	5′-TTCGGCGCGCCGAATCCTTAATTAAGGTGGCCTTCTCCCGTGTGCTG-3′		This work
DspE-CF3	5′-CCTTAATTAAGGATTCGGCGCGCCGAATGCGGCAACGGAACGAACAA-3′	651	This work
DspE-DR4	5′-TACGGTTGTATCAGGGATGG-3′		This work
YecF-AF1	5′-CTGGCATGTTCATATCCATC-3′	705	This work
YecF-BR2	5′-TTCGGCGCGCCGAATCCTTAATTAAGGGAAGTTGTATTCTTTTTATC-3′		This work
YecF-CF3	5′-CCTTAATTAAGGATTCGGCGCGCCGAATCAAAACCCATCGGACATCA-3′	577	This work
YecF-DR4	5′-ATCAGTCTGAAGCCGACTGG-3′		This work
YijC-AF1	5′-ATCGCTGTCGTAGATGCGGG-3′	718	This work
YijC-BR2	5′-TTCGGCGCGCCGAATCCTTAATTAAGGCGGCTCTCCTTAAGTCAAGG-3′		This work
YijC-CF3	5′-CCTTAATTAAGGATTCGGCGCGCCGAATCAGAGGAAAAGTCATGACA-3′	581	This work
YijC-DR4	5′-CGTATCCTGTACATCTCCTG-3′		This work
HrpA-AF1	5′-TTCGGCGCGCCGAATCCTTAATTAAGGATCATTCTGCCGTTATTCGA-3′	593	This work
HrpA-BF2	5′-GCTCTAGAGCGATAAATATCTCCAGTTAAC-3′		This work
HrpA-CF3	5′-GCTCTAGAGCTGTTGTGTAGGTAGTCGACT-3′	577	This work
HrpA-DR4	5′-CCTTAATTAAGGATTCGGCGCGCCGAACTCATCATGGCGCCATTTAT-3′		This work
HrpLRT-F	5′-TGCCTGAAGCTGCCTCTGCT-3′	300	This work
HrpLRT-R	5′-CCATCGATATGCCCCAATTG-3′		This work
HrpART-F	5′-ACTGAATAGCTTTGGCCGCT-3′	194	This work
HrpART-R	5′-GGGACTTTCTAACGCAGCAG-3′		This work
HrpKRT-F	5′-ACCGTATCGGCCTTTGGCCT-3′	580	This work
HrpKRT-R	5′-CTTAACGAGCGTGCGTCCGC-3′		This work
DspERT-F	5′-TTCAGCGTCAGCAATCGGTG-3′	590	This work
DspERT-R	5′-GCCGTTACGATTGACGATCG-3′		This work
YijCRT-F	5′-CAGGATAATGGGTGTCAGAG-3′	634	This work
YijCRT-R	5′-GTAAACAAACCCCTTACCCT-3′		This work
YecFRT-F	5′-AGAATGGTTATTTTCCTGAC-3′	355	This work
YecFRT-R	5′-TAGACTGTTTTGATTTGCTT-3′		This work

showed similar or stronger fluorescence than that of *E. coli* cells containing P*hrpN*/pCPP3042 and P*hrpG*/pCPP3042, was picked and cultured in MM overnight with appropriate antibiotics. Plasmid DNA of pCPP3042 (~31 kb) and recombinant clones of P*Ech3937* (~6–8 kb) were extracted and separated by agarose gel electrophoresis. DNA of recombinant clone of P*Ech3937* was purified from the gel and electroporated into *E. coli* DH5α and *E. coli* DH5α carrying pCPP30421, respectively. The recombinant clones of P*Ech3937* that showed GFP fluorescence in *E. coli* DH5α carrying pCPP3042, but no GFP fluorescence

in *E. coli* DH5α carrying pCPP30421 or *E. coli* DH5α were selected and further characterised as *hrpL* up-regulated recombinant clones. The screening experiment was repeated three times.

Complementary experiments

DNA of pDSK6001 and pRK4151 were electroporated into *Escherichia coli* DH5α carrying pCPP30421 and *hrpL* up-regulated recombinant clones. *Escherichia coli* carrying either pPROBE-AT, pDSK600, or pRK415 was used as the negative controls (Table 3). In addition, DNA of *hrpL* up-regulated recombinant clones was electroporated into the wild-type Ech3937 and Ech131 cells, respectively. DNA of pDSK6001 or pRK4151 was electroporated into the above described Ech131 cells (Table 4). Ech3937 carrying pPROBE-AT and Ech131 carrying either pPROBE-AT, pDSK600 or pRK415 was used as the negative controls. The bacterial cultures were grown in MM and the expression of fluorescence was examined under a fluorescence microscope.

Sequencing and annotating hrpL up-regulated genes

The confirmed *hrpL* up-regulated recombinant clones were end-sequenced using primers pPROBE-AT For/Rev (Table 2) and compared with the Ech3937 genome sequence via ASAP (a systematic annotation package for community analysis of genomes http://asap.ahabs.wisc.edu/asap/ASAP1.htm) to obtain the full-length DNA sequence of each insert (Glasner et al. 2006). The individual promoter and ORF within the insert was obtained from ASAP, in which each gene was assigned a unique ASAP number. The promoters that initiate the transcription of the transcriptional-fused GFP protein in *hrpL* up-regulated recombinant clones were selected as *hrpL* up-regulated promoters, which were designated as *hrpL* up-regulated genes (Table 5). The predicted signal peptides and the subcellular localisations of five HrpL up-regulated proteins in prokaryotes were analysed by SignalP (Emanuelsson et al. 2007).

Comparisons of DNA sequences in promoter regions

Promoter sequences of *hrpL* up-regulated genes were determined from start condon (ATG) and extended from 1 nucleotide 5′ upstream of the −35 motif through 3 nucleotides 3′ downstream of the −10 motif. The extended promoter sequences were aligned via alignX in Vector NTI 9.0. The reverse complement alignment was also generated by using the BCM search Launcher online (http://searchlauncher.bcm.tmc.edu/). The promoter sequences of *hrcC*, *hrpG, hrpB, hrpJ*, and *hrpD* were aligned with the promoter sequences of *hrpL* up-regulated genes. The identified promoter consensus sequences were also compared with the consensus promoter sequence of the Hrp box of *P. syringae* tomato DC3000 (Fouts et al. 2002).

Table 3 The observed GFP fluorescence of *hrp* up-regulated recombinant clones in *E. coli* complementation experiment

Clone ID[a] (gene)[b]	*E. coli*[c]	*E. coli* pCPP3042	*E. coli* pCPP30421	*E. coli* pCPP30421/ pDSK6001	*E. coli* pCPP30421/ pRK4151	*E. coli* pDSK600	*E. coli* pRK415	*E. coli* pDSK6001	*E. coli* pRK4151
pPROBE-AT	−	−	−	−	−	−	−	−	−
P*hrpN*	−	+	−	+	+	−	−	+	+
P*hrpG*	−	+	−	+	+	−	−	+	+
XY123 (*hrpA*)	−	+	−	+	+	−	−	+	+
XY778 (*hrpK*)	−	+	−	+	+	−	−	+	+
XY7108 (*dspE*)	−	+	−	+	+	−	−	+	+
XY78 (*yijC*)	−	+	−	+	+	−	−	+	+
XY453−1(*yecF*)	−	+	−	+	+	−	−	+	+

[a] Names of the clones in the order detected in the experiment.

[b] Gene identifications are based on ASAP annotation and BLAST homologues and accordingly are considered provisional.

[c] +: fluorescence was observed in the bacterial cells.

−: no fluorescence was observed in the bacterial cells.

Table 4 The observed GFP fluorescence of *hrp* up-regulated recombinant clones in Ech3937 complementation experiment

Clone ID (gene)[a]	Ech3937[b]	Ech131	Ech131/pDSK6001	Ech131/pRK4151	Ech131/pDSK600	Ech131/ pRK415
pPROBE-AT	−	−	−	−	−	−
P*hrpN*	+	−	+	+	−	−
P*hrpG*	+	−	+	+	−	−
XY123 (*hrpA*)	+	−	+	+	−	−
XY778 (*hrpK*)	+	−	+	+	−	−
XY7108 (*dspE*)	+	−	+	+	−	−
XY78 (*yijC*)	+	+−	+	+	−	−
XY453-1(*yecF*)	+	+−	+	+	−	−

[a] Gene identifications are based on ASAP annotation and BLAST homologues and accordingly are considered provisional.

[b] +: fluorescence was observed in the bacterial cells.

−: no fluorescence was observed in the bacterial cells.

+−: fluorescence was weaker than +.

Gene knockouts in Ech3937

A crossover PCR strategy was used to knockout the target genes as described previously (Yang et al. 2002). The final PCR fragments containing the upstream side (amplified with primers AF1/BR2) and the downstream side (amplified with primers CF3/DR4) of the target genes *hrpL, hrpK, dspE, yecF, yijC* and *hrpA* (Table 2) were cloned into pGEM-T Easy (Promega, MI), respectively, as pGEMT-L, pGEMT-K, pGEMT-E, pGEMT-F, pGEMT-C, and pGEMT-A (Table 1). A kanamycin cassette released from pGEMT-3 by *AscI* was cloned into the *AscI* site of the final PCR fragment in the above plasmids, resulting in mutagenic constructs as pGEMT-LK, pGEMT-KK, pGEMT-EK, pGEMT-FK, pGEMT-CK, and pGEMT-AK (Table 1). Target gene fragments disrupted with kanamycin cassette were released by *Not*I from these mutagenic constructs and cloned into pBSK1, resulting in pBSK-LK, pBSK-KK, pBSK-EK, pBSK-FK, pBSK-CK, and pBSK-AK (Table 1), which were further electroporated into the wild-type Ech3937. Plasmid curing and marker exchange were accomplished by growing transformants on low phosphate medium supplemented with kanamycin (Ried and Collmer 1987). Kanamycin-resistant and chloramphenicol-sensitive colonies were determined as mutant strains Ech131, Ech132, Ech133, Ech134, Ech135 and Ech136 (Table 1).

Wild-type Ech3937 or its mutant strains were cultured in 5 ml LB broth at 28°C overnight with or without antibiotics. The genomic DNA was extracted from the wild-type Ech3937 or its mutant strains, respectively, with a MasterPure DNA purification kit (Epicentre Biotechnologies). The PCR fragments amplified from the genomic DNA of the wild-type and the mutant strains with the specific gene primers HrpL-AF1/DR4, HrpK-AF1/DR4, DspE-AF1/DR4,

Table 5 Genes that were identified to be up-regulated by *hrpL* in Ech3937

Clone ID[a]	ASAP ID[b]	Homologous gene[c] (organism)	Product/ Functions
XY123	19593	*hrpA* (*Erwinia carotovora*)	type III protein secretion system pilus subunit
XY778, XY7101	19004	*hrpK* (*Pseudomonas syringae*)	type III protein secretion system putative translocator
XY7, XY143, XY7108	19012	*dspE* (*E. amylovora*)	type III secretion system effector protein
XY78	20422	*yijC* (*E. coli*)	DNA-binding transcriptional regulatory protein (TetR family)
XY453-1	20514	*yecF* (*E. coli*)	Unknown function

[a] Names of clones in the order detected in the experiment. Several clones were designated to one gene.

[b] Each gene has a unique ASAP number in *D. dadantii* 3937 genome annotation website ASAP (a systematic annotation package for community analysis of genomes (http://asap.ahabs.wisc.edu/asap/ASAP1.htm).

[c] Gene identifications are based on ASAP annotation and BLAST homologues and accordingly are considered provisional.

YecF-AF1/DR4, YijC-AF1/DR4 and HrpA-AF1/DR4 (Table 2), respectively, were cut from the gel, cloned into pGEM-T Easy, end-sequenced, and compared with Ech3937 genomic sequences or the kanamycin cassette sequences with Vector NTI (Invitrogen, CA). The locations of kanamycin cassettes-within the cloned specific PCR fragments of the mutant strains, respectively, were further confirmed by sequencing with primers Kan-2 FP-1 or Kan-2 RP-1 (Table 2), which determined the locations of kanamycin cassette into the genome of the mutant strains.

RNA isolation, quantification, reverse transcription polymerase chain reaction (RT-PCR)

Cells of the wild-type Ech3937 and its mutant stains were collected and adjusted to an OD_{600} of 0.01. One hundred and fifty microlitre aliquots of each cell culture were added into 50 ml MM liquid medium, and grown at 28°C under constant shaking until cell density reached OD_{600}=1.0. Total RNA was extracted from the bacterial cells with PureYield™ RNA Midiprep System (Promega, WI). RNA was treated with Turbo DNA-free™ DNase (2 U μl^{-1}) (Ambion, TX) to ensure that the RNA preparation was DNA-free. The quantity of the isolated RNAs was determined by electrophoresis. The expression of *hrpL, hrpA, hrpK, dspE, yecF, and yijC* in Ech3937 and Ech131 was analysed by RT-PCR with gene specific primers RT-F/R (Table 2), using the AccessQuick™ RT-PCR System per manufacturer's instructions (Promega, WI). The expression of *hrpA, hrpK, dspE, yecF, and yijC* in Ech136, Ech132, Ech133, Ech134, and Ech135 was also analysed by RT-PCR. 16S Ribosome RNA was used as the control.

Virulence assays on host plants

A colony of wild-type Ech3937 or its mutants was grown in 5 ml LB with shaking (180 rpm) at 28°C overnight. The bacterial cells were collected and resuspended in 50 mM phosphate buffer (pH 7.4) to a concentration of 10^6 CFU ml^{-1}. The bacterial suspension (0.05 ml) was infiltrated into fully-expanded leaves of 2–3 month old African violet (*Saintpaulia ionanthai*) cv. Rosalie using the syringe–infiltrated method (Yang et al. 2002). Five plants with a total of ten leaves were inoculated with each strain for virulence assays. The inoculated plants were placed in a growth chamber with 95% relative humidity and a photoperiod of 16 h at 28°C, as described previously (Yang et al. 2002). The progress of the infection was observed every day for 1 week. The maceration area caused by bacterial infection was precisely measured with ASSESS, which is an image analysis software for plant disease quantification (The American Phytopathological Society Press, Minnesota).

Results

Identification of hrpL up-regulated genes

A GFP-based *E. coli* promoter-probe system was used to screen for *hrpL* up-regulated genes from Ech3937. No fluorescence was observed from *E. coli* DH5α containing either pPROBE-AT, P*hrpN*, P*hrpG*, pCPP3042 or pCPP30421 alone in MM liquid medium. Strong fluorescence was observed from *E. coli* DH5α cells containing P*hrpN*/pCPP3042, or P*hrpG*/pCPP3042, but not from *E. coli* DH5α cells containing P*hrpN*/pCPP30421, or P*hrpG*/pCPP30421. From the Ech3937 pPROBE-AT library (P*Ech3937*), ca. 20,000 clones (36% of total library clones) were screened. Five *hrpL* up-regulated recombinant clones showed similar or stronger fluorescence than that of *E. coli* DH5α carrying P*hrpN*/pCPP3042 or P*hrpG*/pCPP3042. These five recombinant clones showed no fluorescence in *E. coli* DH5α alone and *E. coli* carrying pCPP30421 (Table 3).

A stronger fluorescence was observed when either high copy number pDSK6001 or low copy number pRK4151 was present in those *E. coli* cells carrying pCPP30421 and the five *hrpL* up-regulated recombinant clones, respectively (Table 3). No fluorescence in *E. coli* carrying five *hrpL* up-regulated clones and either pDSK600 or pRK415 was detected. All five *hrpL* up-regulated recombinant clones tested in *E. coli* complementation experiments were further confirmed in Ech3937 cells (Table 4). As a negative control, no fluorescence was observed in Ech131 containing five *hrpL* up-regulated recombinant clones and either pDSK600 or pRK415, respectively. A stronger fluorescence was observed when either pDSK6001 or pRK4151 was present in those Ech131 cell containing five *hrpL* up-regulated recombinant clones, respectively (Table 4). Similarly, a higher

fluorescence was observed in wild-type Ech3937 carrying five *hrpL* up-regulated recombinant clones compared to Ech131 containing the same clones (Table 4), indicating that this result was consistent with the complementation experiment performed in *E. coli* cells, and five *hrpL* up-regulated recombinant clones were HrpL-dependent.

Gene sequence and annotation analysis indicated that five *hrpL* up-regulated genes of Ech3937 include a T3SS apparatus component (*hrpA*; ASAP19593), a putative T3SS helper protein (*hrpK*; ASAP19004), a T3SS effector (*dspE*; ASAP19012), a DNA-binding transcriptional regulatory protein (*yijC*; ASAP20422), and a gene with unknown function (*yecF*; ASAP20514) (Table 5).

Distinct fluorescence among Ech3937 and Ech131 cells was detected in five *hrpL* up-regulated genes which can be classified into two groups. In group 1 (including *hrpA, hrpK* and *dspE*), Ech131 containing *hrpA*, *hrpK*, and *dspE* in pPROBE-AT have no detectable fluorescence of GFP at 12 h in MM (Table 4). In contrast, Ech3937 carrying the same constructs have strong detectable fluorescence of GFP. *yijC* and *yecF* were classified in group 2, in which Ech131 carrying *yijC* and *yecF* in pPROBE-AT still have lower detectable fluorescence of GFP compared to Ech3937 carrying the same constructs, respectively (Table 4).

Putative consensus sequences were observed in the promoter regions of the *hrpA, hrpK*, *dspE,* and *yijC*, but not in promoter region of *yecF* (Fig. 1). The consensus sequences showed similarity with the Hrp box of *P. syringae* tomato DC3000.

The detection of mRNA expressions

The *hrpL, hrpA, hrpK*, and *dspE* RNA were not detectable in Ech131 compare to the wild-type Ech3937, and *yijC* and *yecF* RNA were decreased in Ech131 (Fig. 2). The *hrpK, dspE, yecF, yijC*, and *hrpA* RNA were not detectable in Ech132, Ech133, Ech134, Ech135, and Ech136 by RT-PCR, respectively (date not shown).

Virulence assay

Ech131, Ech132, Ech133 and Ech136 had a reduced virulence in African violet as compared to wild-type Ech3937 (Fig. 3). Ech131 and Ech136 had no virulence in African violet because the HrpL is a sigma factor regulating other effector proteins and HrpA is the main component of Hrp pilus. Ech132 and Ech133 had an approximate eight-fold reduction of maceration compared to the wild-type Ech3937 in African violet. This suggests that mutated Hrp putative translocator HrpK and an essential effector protein DspE caused dramatically reduced pathogenicity of Ech3937. Ech134 and Ech135 had no significant reduced maceration compared to the wild-type strain in African violet. This suggests that some mutated HrpL-dependent genes, such as *yijC* and *yecF*, still had a higher virulence compared to the wild-type Ech3937 in African violet.

Discussion

The GFP-based *E. coli* promoter-probe system was used to screen 61 plant up-regulated genes of Ech3937 identified in an *in vivo* expression technology (IVET) assay (Yang et al. 2004); only T3SS gene *hrpB*, but no other genes, was detected to produce stronger GFP fluorescence, suggesting that this screen system was specific for identifying *hrpL* up-regulated genes in Ech3937. It has been reported that an elevated production of *hrpL* from plasmids may cause atypical expression of some genes, and strong constituting plasmid promoters can promote the

Fig. 1 The analysis of consensus sequences in promoter regions of *hrpL*-regulated genes. The analysis of alignment and phylogenetic tree were performed using alignX (Vector NTI 9.0). The consensus sequence was conducted via alignment of the promoter sequences of *hrpJ-hrcU* operon, *hrpVT-hrcC-hrpGF* operon, *hrpED-hrcJ-hrpBA* operon and four detected *hrpL*-regulated genes in this study

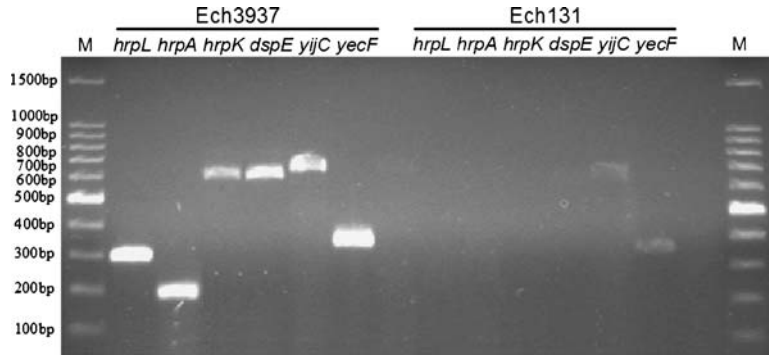

Fig. 2 Reverse transcription polymerase chain reaction (RT-PCR) of genes differentially expressed between the wild-type Ech3937 and Ech131. rRNA was detectable in Ech3937 and Ech131 in this RT-PCR condition. The *hrpL, hrpA, hrpK,* and *dspE* RNA were not detectable in Ech131 compared to Ech3937, and *yijC* and *yecF* RNA were decreased in Ech131

production of sufficient fusion protein, resulting in resurrecting transcriptional silent or *hrpL*-independent ORFs (Chang et al. 2005). However, in the complementation experiments in this study, the similar GFP fluorescence was detected in *E. coli* or Ech131 cells carrying five *hrpL* up-regulated recombinant clones with high copy number *hrpL* plasmid and low copy number *hrpL* plasmid, respectively (Tables 3 and 4). Results showed that the constituting plasmid promoter has less effect on atypical expression of *hrpL* up-regulated genes in this study. The consistent fluorescence for five *hrpL* up-regulated recombinant clones in this study showed that this GFP-based *E. coli* promoter-probe system for screening *hrpL* up-regulated genes was reliable.

Five *hrpL* up-regulated genes include known *hrp*-related virulence factors *dspE*, the translocator *hrpK*, and *yijC* involved in catabolic metabolism in other bacteria such as *Pectobacterium carotovora, E. amylovora*, and *P. syringae,* and *E. coli*. YijC contains a conserved TetR domain (*E* value, 1e-05). In addition, YijC contains a consensus T3SS targeting pattern in the first 50 amino acids of the N-terminal region but showed no homology to other Hrp outer proteins (Hop) reported (Collmer et al. 2002). In *E. coli*, the FabR transcription factor, which has 64% similarity with YijC, regulates *fabA* and *fabB* genes involved in unsaturated fatty acid biosynthesis. Thus, the *hrpL* of *D. dadantii* is probably involved in the bacterial catabolic metabolism and pathogenicity during the bacterial infection of the host plant.

hrpA of *P. syringae* and *Pectobacterium carotovora* encodes a major structural component of the T3SS pilus (Collmer et al. 2002; Chatterjee et al. 2002). HrpK is a putative translocator of T3SS in *P.*

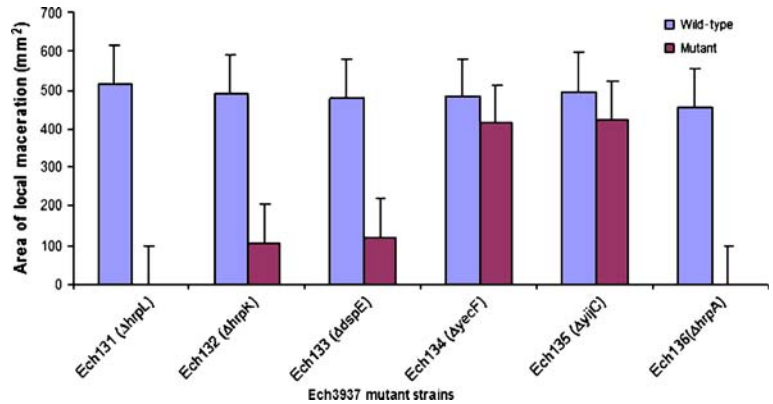

Fig. 3 Local lesion maceration by the wild-type Ech3937 and its mutants on African violet cv. Rosalie. The maceration area was precisely measured with ASSESS, an image analysis software for plant disease quantification as mentioned in Materials and methods. Data are an average of ten leaves in five plants for each bacterial strain. The maceration areas were statistically different after student *t*-test, $P<0.05$

syingae (Petnicki-Ocwieja et al. 2005). The *dspE* gene of *E. amylovora* is homologous to *avrE* of *P. syringae* pv. *tomato* (Bogdanove et al. 1998). DspE of *E. amylovora* is secreted via the Type III secretion pathway in *E. amylovora* cells grown in a *hrp* gene-inducing MM (Bogdanove et al. 1998). In this study, the RNA of *hrpA, hrpK,* and *dspE* was not detectable in Ech131 (Fig. 2), and the putative consensus sequences observed in the promoter regions of the *hrpA, hrpK*, and *dspE* were higher, similar to the consensus sequences of *hrpJ-hrcU* operon, *hrpVT-hrcC-hrpGF* operon, *hrpED-hrcJ-hrpBA* operon, and with the Hrp box of *P. syringae* pv. *syringae* (Fig. 1). Thus it is not surprising that *hrpA, hrpK*, and *dspE* were tightly regulated by *hrpL* in *D. dadantii*. In the experiments reported here, the *hrpA*, *hrpK* and *dspE* mutants of Ech3937 had reduced virulence on African violet (Fig. 3), demonstrating that *hrp* genes are critical virulence factors in *D. dadantii*.

yijC and *yecF* were detected to be regulated by *hrpL*. A putative consensus sequence was unexpectedly found at the promoter region of *yijC,* which is in the same location as in *hrpA*, *hrpK* and *dspE* (Fig. 1). This suggests there is consensus sequence between HrpL-dependent genes, if they have it. The consensus promoter sequence of *yijC* is similar to that of *hrpK* (Fig. 1), which locates in exchangeable effector locus (EEL) of Hrp Pathogenicity Island (PAI) of *P. syringae* (Alfano et al. 2000). The *hrpL* up-regulated YecF encodes a putative protein with unknown function. Interestingly, no apparent consensus sequence was observed in the promoter region of *yecF*. The biological function and regulatory mechanism of *yijC* and *yecF* of Ech3937 remains to be further investigated.

Distinct fluorescence was observed among cells of Ech131 carrying *hrpA, hrpK, dspE, yijC* and *yecF* in the complementary assays (Table 4). The reason for these different gene expression patterns is unclear. There are still expressions of *yijC* and *yecF* in Ech131 suggesting that HrpL may regulate the transcription of *yijC* and *yecF*. In this study, the RNA of *yijC* and *yecF* was decreased in Ech131 compared to Ech3937 (Fig. 2), indicating they are not tightly HrpL-dependent genes in Ech3937. It is noted that *yijC* was involved in the metabolism functions in *E. coli*, and *yecF* has an unknown function. The importance of YijC and YecF in the Ech3937-plant interaction was shown in the virulence assay in which the *yijC* and *yecF* mutant had a reduced virulence in African violet (Fig. 3). One possible explanation is that, although YijC and YecF are regulated by HrpL, the functions of these two gene products might be required for pathogenic bacteria to cope effectively with the nutrients for survival in a hostile environment within the host (Wassenaar and Gaastra 2001). Thus, the YijC and YecF may be responsible for routine metabolic functions or the parasitic fitness in Ech3937 and are required to be expressed in cells.

hrpA, hrpK, and *dspE* are virulence genes, which are directly involved in damaging the host cells or in regulating virulence genes (Wassenaar and Gaastra 2001). *hrpA, hrpK*, and *dspE* genes are specifically used by the bacterium for its pathogenicity, and only expressed as needed for infection of host plants. Indeed, compared with a putative housekeeping gene *mrp* (99.47% Ech3937 cells expressing fluorescence), only a proportion of *pelD* in Ech3937 cells (69%) was expressed under an homogenous MM inducing condition (Peng et al. 2006). These phenomena indicate that plant pathogenic *D. dadantii* potentially regulates the expression of sets of genes as a necessary requirement during survival within the host. These data indicate that there are different regulatory pathways among *hrpL* dependent genes at the population level during *D. dadantii* infection of the host plant.

Recently, *hrpN*, which was also up-regulated by *hrpL*, was shown to be essential for cell-to-cell aggregation, which plays an important role in plant-bacteria interactions (Yap et al. 2006). In conclusion, it is suggested that *hrpL* is involved in a regulatory cascade of virulence and pathogenicity in Ech3937. A further investigation of the biological functions of these newly identified *hrpL* up-regulated products and the regulatory network between *hrpL* and these *hrpL* up-regulated genes in Ech3937 is underway.

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