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Exploring the functions of genes co-regulated with the hrpn gene of the corn pathogen *Erwinia* (*Pantoea*) *stewartii*

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Abstract

The 28 kb wts cluster of *Erwinia stewartii* consists of eight complementation groups including wtsE, wtsN, wtsF, wtsD, wtsA, wtsC, wtsI and wtsB. wtsA encodes a positive regulatory protein whereas the product of hrpN (wtsN) is a hypersensitive response eliciting protein. wtsE encodes an effector protein which causes water-soaking in corn. However, the functions of the wts genes in the remaining complementation groups were not known. Production and secretion of harpinEs by wts mutants was used to explore the functions of wtsF, wtsB, wtsD and wtsI. The functions of wtsF, wtsB, and wtsD were found to be export-related whereas wtsI was concluded to be regulatory.

Introduction

The ability of plant pathogenic bacteria to elicit the hypersensitive response (HR) in resistant host or non-host is correlated with their ability to cause disease in susceptible host. Both of these capabilities are controlled by a group of genes called hrp genes (for hypersensitive response and pathogenicity) (Lindgren *et al.,* 1986, Willis *et al.,* 1991). The hrp genes consist of large clusters which have many complementation groups. Usually, one gene in the cluster encodes a heat-stable, protease-sensitive, and glycine-rich protein called harpin, which is required for elicitation of HR in tobacco and other non-host plants (Wei *et al.,* 1992). The remaining genes in hrp clusters are needed for the synthesis of possible "disease specific" extracellular pathogenicity factors, secretion of exrtracellualr proteins, and hrp gene regulation.

The hrp clusters of many plant pathogenic bacteria are known to have genes encoding proteins for regulatory and export functions. For example nine Hrp proteins that have export functions have been found in *Erwinia amylovora*. These proteins constitute the Sec-independent type III secretion pathway that is used for secretion of harpins and possibly other extra-cellular proteins (Bogdanove *et al.,* 1998).

The first regulatory protein , HrpS, was found in *Pseudomonas syringae* pv. *phaseolicola* (Grimm & Panopoulos, 1989). HrpS is a 34.5 kDa protein similar to several members of the NtrC-family of regulatory proteins, which is present in enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* etc. HrpS interacts with HrpR and õ 54 to activate hrpL promoter. hrpL encodes an alternate sigma factor, which in turn activates the remaining hrp operons of *P. syringae* pv. *phaseolicola* (Grimm *et al.,* 1995).

Erwinia stewartii has been found to have a 28kb pathogenicity/wts (for water soaking) gene cluster which is required for lesion formation and wilting on corn. This cluster consists of complementation groups wtsE, wtsN, wtsF, wtsD, wtsA, wtsC, wtsI and wtsB (Coplin *et al.*, 199a, Coplin *et al.,* 199b). Since the wts cluster of *Erwinia stewartii* has a gene for harpin production (Frederick *et al*., 2001, Ahmad *et al.,* 2005) and it hybridizes to the hrp clusters of *E. amylovora* and *P. syringae* pv. *phaseolicola*, we expected it to also provide the Hrp secretion and regulatory functions. The wts cluster of *E. stewartii* has a hrpS homolog, wtsA, which positively regulates other wts genes (Frederick *et al*., 1993). However, the functions of the remaining complementation groups were unknown. In this paper, we explore the functions of wtsF, wtsB, wtsD, and wtsI to determine which of these complementation groups/ genes are involved in extra-cellular protein production, secretion or regulation.

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Materials and Methods

Bacterial strains used in this study are listed in Table 1. All *E. stewartii* strains were derived from DC 283 or DC 356, which are spontaneous nalidixic acid-resistant (Nalr) and rifampicin-resistant (Rifr) mutants of wild-type strain SS 104 (Coplin *et al.*, 1981), respectively. pRF 205 was mobilized from *E. coli* HB101 into *E. stewartii* strains containing mutations in wts F, wts B, wts D, and wts I complementation groups in triparental matings using the helper plasmid pRK 2013:: Tn7 (Coplin *et al.,* 1986). Culture media, growth of bacteria and mating conditions for *E. stewartii* have been described previously (Coplin *et al.*, 1986). The following antibiotics were used in the media in the amounts indicated (µg/ml) tetracycline, 20; ampicillin, 100; and nalidixic acid, 20. The inducing medium (IM) (Majerczak and Coplin, unpublished) consisted of 100 mM 2-N-morpholinoethanesulfonic acid (MES; Sigma Chemical, St. Louis, MO); 2 mM (NH4)2 SO4; 0.1 % casamino acids (Difco, Detroit, MI); 1 mM potassium phosphate (pH 7.2); 1 % sucrose; and 1 mM MgSO4 .The medium was adjusted to pH 5.5 with NaOH. Sucrose and MgSO4 were added after autoclaving.

Protein manipulations and western blotting.

HarpinEs was extracted from DC 283(pRF 205), and *E. stewartii* strains carrying mutations in complementation groups wts F, wts B, wts D, and wts I (each containing pRF 205). For this purpose, the cells were first grown in 100 ml LB (plus antibiotics) at 280C to A540 = 0.8. Next, the cells were pelleted and washed in 10 ml of IM, resuspended in 100 ml of the same medium, and incubated with shaking for 24 hrs. Then, the cells were pelleted and disrupted by sonication (Wei *et al.*, 1992). The sonicate was boiled for 10 min. and centrifuged to remove the denatured proteins. The resulting cell free elicitor preparation (CFEP) contained partially purified protein harpinEs. To concentrate harpin, the CFEP was mixed with equal volume of 10 % TCA, iced for 15 min., and centrifuged at 20,400 x g. The pellet was dissolved in 200 µl sample denaturation buffer.

For Western blotting, the CFEPs of different strains were subjected to PAGE (Sambrook et al., 1989) and electroblotted on ImmunoSelect (GIBCO BRL, Gaithersburg, MD) nitrocellulose membranes. The filters were probed with anti-harpinEs serum, and immunodetection of bands was performed with a rabbit alkaline phosphatase-conjugated secondary antibody (Protoblot R II AP system-kit) as described by the manufacturers (Promega, Madison, WI) instructions.

HR assay in tobacco plants.

Tobacco plants (*Nicotiana tabacum* L. var. Wisconsin) were grown under greenhouse conditions and then transferred to a controlled environment chamber maintained at 280C, 90% relative humidity, 16 h light and 8-h dark cycle. Bacteria were prepared by pelleting overnight cultures grown in IM or LB and resuspending the cells in 10 mM phosphate buffer at 5x108 cells/ml (A540 = 0.52). Tobacco leaves were infiltrated with bacteria or CFEPs by pricking them with a dissecting needle and then pressing the open end of a 3 ml disposable plastic transfer pipet (Denville Scientific, Denville, NJ) against the lower leaf surface and forcing the inoculum into the wound (Bauer *et al.,* 1994). The margins of the water-soaked infiltrated areas were marked and the plants were rated for HR development at 24 h. The percent of HR for different strains was calculated as the proportion of the infiltrated area showing necrosis.

Results and Discussion

Because *E. Stewartii* neither produces detectible quantities of harpin nor does it give HR under normal assay conditions (Ahmad *et al.*, 2001), we first introduced plasmid pRF 205 into the wtsF, B, D, and I mutant strains. pRF205 contains wtsA, cloned behind the vector P promoter (Frederick *et al*., 1993), so it enhances harpinES production and the expression of other Wts functions, thereby enabling wild-type strains to give an HR.

When live cells of the wild-type strains DC283 (pRF205) and the wts mutants containing pRF205 were grown in either IM or LB, and infiltrated into tobacco and Datura, only DC283 (pRF205) produced typical HR symptoms within 24 h, whereas the wts mutants containing pRF205 did not give any HR, However when the cells were disrupted by sonication, a strong HR was observed with CFEP from DC283 (pRF205) as well as with those from wtsF, wtsB, and wtsD mutants, but not from the hrpN and wtsI mutants (Table 2). Moreover, the HR correlated with the presence of intracellular harpin in the CFEPs as shown by western blots (Fig. 1)

The model for hrp gene regulation in *P. syringae* pv. *phaseolicola* suggested that the *E. stewartii* wts cluster might include several positive regulatory genes in addition to wtsA. In this study, we found that a wtsI mutant was HR- and non-pathogenic and could not synthesize harpin intracellularly. This pleiotropic phenotype indicates that it is defective in the secretion and synthesis of harpin and other pathogenicity proteins and wtsI may therefore encode a regulatory protein. Preliminary

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sequence analysis indicates that WtsI is related to HrpL (Coplin and Majerczak, unpublished) and we are postulating that it acts between wtsA and the individual operons. wtsC mutants have a similar phenotype and may also be regulatory (Coplin and Stover, unpublished).

The observation that the cells of *E.stewartii* strains containing mutations in wtsF, wtsB, and wtsD did not cause an HR in tobacco and accumulated harpin intracellularly suggests that the genes in these complementation groups encode proteins needed for the export of harpinEs. A similar phenotype has been reported for harpin secretion mutants of other bacteria. For example, the export of harpinPss of *P.syringae* pv. *syringae* is dependent on hrpH (He *et al*., 1993), hrpW and hrpY (Huang *et al*., 1995) and possibly other genes. However, it is not known whether wtsF, wtsB, and wtsD function directly in export or regulate the export process. It may be that some genes in these complementation groups encode proteins that are the constituents of the export system while others synthesize proteins having export-related regulatory roles. Since hrpN mutants of *E. stewartii* are still pathogenic, the genes in the wtsF, wtsB, and wtsD complementation groups are probably used not only for the secretion of harpinEs but for other pathogenicity proteins as well, because mutants in these groups are completely non-pathogenic. Recent studies (Ham *et al*., 2005) show that wtsE encodes an effector protein (WtsE) which causes water-soaking in corn leaves. wtsF is downstream of wtsE in the same operon although it has its own ó70 promotor. By virtue of its small size (15.6 kDal) and physical properties WtsF (protein product of wtsF) is predicted to be a chaperone for WtsE (Coplin .2005, unpublished). As wtsF mutants retain significant pathogenicity, it appears that WtsF is not required for WtsE translocation. However, the amount of WtsE present in both the cell pellets and culture supernatants of wtsF mutants is greatly reduced suggesting that the major role of this chaperone is to stabilize WtsE inside the bacterium and protect it from degradation (Ham *et al.,* 2005).

Table 1. Bacterial strains and plasmids

Bacterial strains/plasmids	Relevant characteristics[a]	Reference or source
Bacterial strains:		
Erwinia (*Pantoea*) *stewartii*		
DM 760	DC 283 *hrpN* 189:: Tn*5*	Frederick *et al*., 2001
DC 283	SS 104 Nal^r	Coplin *et al*., 1981
MEX 101	DC 283 *wtsF* 101::Tn*3*HoHo1	This lab
MU 141	DC 283 *wtsB*141::Mu *kan* pf7701	McCammon *et al*., 1985
MEX 105	DC 283 *wtsI*105::Tn*3*HoHo1	This lab
DM 4031	DC 283 *wtsD*4031::Tn*5*	This lab
E. coli HB 101	*thr leu thi recA hsdR hsdM pro*	Boyer and Dussoix, 1969
Plasmids:		
pRK 2013::Tn7	ColE1 mob^+ Sm^r Sp^r Tp^r kan::Tn*7*	Dennis Dean[b]
pRF 205	1.8 kb *HindIII* fragment containing P*lac*:: *wtsA* in pVK100 Cloning vector, Ap^r	Frederick *et al*., 1993

[a] Nal^r, Sm^r, Tp^r, Sp^r : resistant to naladixic acid, streptomycin, trimethoprim, and spectinomycin respectively.
[b] Verginia Polytechnic Institute and State University, Blacksburg.

Table 2. HR assay in tobacco leaves of cell-free elicitor preparations (CFEPs) from the wild-type strains DC283 (pRF205) and various *wts* mutants.

Strains	**Complementation. group**	**% HR[b] by CFEPs[a]**			
		1[c]	2[c]	3[c]	Mean ± S.D
DC283 (pRF205)	*wts+*	80	95	nt	87 ± 10
DM760 (pRF205)	*hrpN*	0	0	0	0
MEX101 (pRF205)	*wtsF*	62	54	92	69 ± 20
MU141 (pRF205)	*wtsB*	58	85	45	62 ± 20
DM4031 (pRF205)	*wtsD*	97	82	nt	89 ± 10
MEX105 (pRF205)	*wtsI*	3	0	0	1 ± 2

[a] CFEPs were extracted from the respective strains grown overnight at $28^{O}C$ in IM (pH5.5) according to Wei et al. (1992) and infiltrated into the panels of tobacco leaves. [b] % HR was determined by estimating the percentage of the infiltrated area that was necrotic at 24 h. [c] Each column represents an independent experiment. Each value is the mean of at least three replicates and nt = not tested.

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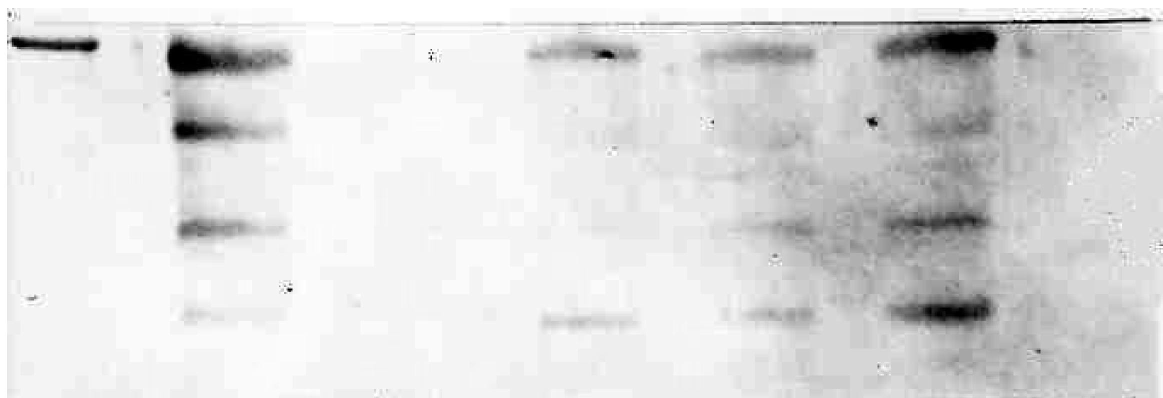

Fig. 1. Immunoblot analysis of harpin production by pRF205-containing *wtsF, wtsB, wtsD,* and *wtsI* mutants. $Harpin_{Es}$ is indicated by the arrow. The low molecular weight cross-reacting bands are due to $harpin_{Es}$ degradation products. 1 = $harpin_{Es}$, 2 = DC283 (pRF205) wts^{+} , 3 = DM760(pRF205) *wtsN*, 4 = MEX101 (pRF205) *wtsF* , 5 = MU141 (pRF205) *wtsB* , 6 = DM4031 (pRF205) *wtsD* , 7 = MEX105(pRF205) *wtsI*. The gel has been cropped at the well.

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