Short communication

The fire blight pathogen *Erwinia amylovora* requires the *rpoN* gene for pathogenicity in appleLAURA S. RAMOS^{1,2}, BRIAN L. LEHMAN³, JUDITH P. SINN¹, EMILY E. PFEUFER^{1,2}, NOEMI O. HALBRENDT^{1,3} AND TIMOTHY W. MCNELLIS^{1,*}¹Department of Plant Pathology & Environmental Microbiology, The Pennsylvania State University, University Park, PA 16802, USA²Graduate Degree Program in Plant Pathology, Department of Plant Pathology & Environmental Microbiology, The Pennsylvania State University, University Park, PA 16802, USA³The Pennsylvania State University Fruit Research and Extension Center, Biglerville, PA 17307, USA**SUMMARY**

RpoN is a σ^{54} factor regulating essential virulence gene expression in several plant pathogenic bacteria, including *Pseudomonas syringae* and *Pectobacterium carotovorum*. In this study, we found that mutation of *rpoN* in the fire blight pathogen *Erwinia amylovora* caused a nonpathogenic phenotype. The *E. amylovora* *rpoN* Tn5 transposon mutant *rpoN*1250::Tn5 did not cause fire blight disease symptoms on shoots of mature apple trees. In detached immature apple fruits, the *rpoN*1250::Tn5 mutant failed to cause fire blight disease symptoms and grew to population levels 12 orders of magnitude lower than the wild-type. In addition, the *rpoN*1250::Tn5 mutant failed to elicit a hypersensitive response when infiltrated into nonhost tobacco plant leaves, and *rpoN*1250::Tn5 cells failed to express HrpN protein when grown in *hrp* (hypersensitive response and pathogenicity)-inducing liquid medium. A plasmid-borne copy of the wild-type *rpoN* gene complemented all the *rpoN*1250::Tn5 mutant phenotypes tested. The *rpoN*1250::Tn5 mutant was prototrophic on minimal solid and liquid media, indicating that the *rpoN*1250::Tn5 nonpathogenic phenotype was not caused by a defect in basic metabolism or growth. This study provides clear genetic evidence that *rpoN* is an essential virulence gene of *E. amylovora*, suggesting that *rpoN* has the same function in *E. amylovora* as in *P. syringae* and *Pe. carotovorum*.

Fire blight is a very well-known plant bacterial disease, not only because it is the most economically important and destructive bacterial disease in apples and pears around the world, but also because it was the first disease of plants that was demonstrated to be caused by bacteria (Arthur, 1885; Burrill, 1880; van der Zwet *et al.*, 2012). The causal agent of fire blight is *Erwinia amylovora* and the disease is called fire blight because the infected parts of

the tree look as if they have been burned (Coxe, 1817; van der Zwet *et al.*, 2012). The most important and characteristic sign of fire blight disease is bacterial ooze (van der Zwet *et al.*, 2012). Fire blight has been a recognized disease problem for over 200 years (Denning, 1794), and the disease continues to cause major sporadic losses, such as a \$42 million loss in the state of Michigan, USA, in 2000 (Michigan Rotational Survey, 2001). Unfortunately, fire blight threatens to be more economically important in the future, as the disease is still spreading around the world (Vanneste, 2000).

In recent decades, the development of molecular genetics techniques has facilitated the identification of genetic factors that contribute to the ability of *E. amylovora* to cause fire blight disease. Three main classes of virulence genes have been found: *ams* (amylovoran synthesis), *hrp* (hypersensitive response and pathogenicity) and *dsp* (disease-specific). The *ams* genes are involved in the biosynthesis of the exopolysaccharide amylovoran (Bugert and Geider, 1995). The *hrp* genes are required to trigger the hypersensitive response (HR) defence reaction in nonhost plants and are necessary for pathogenicity in susceptible hosts. In *E. amylovora*, *hrpL* is necessary for the regulation and expression of most *hrp* genes (Wei and Beer, 1995), *hrcC* and *hrcA* encode structural components of the type III protein secretion system (T3SS) (Kim *et al.*, 1997), and *hrpN* and *hrpW* encode proteins secreted via the T3SS that contribute to *E. amylovora* pathogenicity and the HR in nonhost leaves (Gaudriault *et al.*, 1998; Kim and Beer, 1998; Wei *et al.*, 1992). The *dspA/E* gene is required specifically for the fire blight disease process (Bogdanove *et al.*, 1998; Gaudriault *et al.*, 1997) and the DspA/E protein is translocated into host cells by the T3SS (Bocsanczy *et al.*, 2008). HrpN assists in the translocation of DspA/E protein into the plant cell (Bocsanczy *et al.*, 2008).

In an effort to identify additional genetic factors critical for *E. amylovora* virulence, we mutated *E. amylovora* strain HKN06P1 (Table 1; Lee *et al.*, 2010) using an engineered Tn5 transposon (EZ-Tn5<R6K-yoriKan-2>Transposome Kit, Epicentre, Madison, WI, USA) and screened the mutants for loss of virulence on detached immature apple fruit (Lee *et al.*, 2010; Sinn *et al.*, 2008). The ability

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Table 1 Plasmids and bacterial strains used in this work.

Plasmid	Description	Source, reference
pCR2.1	PCR cloning vector without an insert	TOPO TA Kit, Invitrogen
EZ-Tn5- <i>rpoN</i>	Plasmid rescued from <i>rpoN</i> 1250::Tn5	This work
pCR2.1- <i>rpoN</i>	<i>rpoN</i> cloned in pCR2.1	This work
Bacterial strain		
<i>Erwinia amylovora</i> strain HKN06P1	Wild-type strain	Lee <i>et al.</i> (2010)
<i>hrcC</i> 118::Tn5	<i>E. amylovora</i> HKN06P1 derivative carrying a Tn5 transposon insertion in the <i>hrcC</i> gene	This work
<i>porF</i> auxotroph	<i>E. amylovora</i> HKN06P1 derivative carrying a Tn5 transposon insertion in the <i>porF</i> gene	This work
<i>rpoN</i> 1250::Tn5	<i>E. amylovora</i> HKN06P1 derivative carrying a Tn5 transposon insertion in the <i>rpoN</i> gene	This work
<i>rpoN</i> 1250::Tn5(pCR2.1)	<i>rpoN</i> 1250::Tn5 carrying pCR2.1	This work
<i>rpoN</i> 1250::Tn5(pCR2.1- <i>rpoN</i>)	<i>rpoN</i> 1250::Tn5 carrying pCR2.1- <i>rpoN</i>	This work

of *E. amylovora* to grow and cause disease symptoms in immature apple fruits is a robust indicator of virulence in apple trees (Lee *et al.*, 2010). One virulence-defective *E. amylovora* mutant had a Tn5 insertion in the *rpoN* gene (Fig. 1). This mutant was named *rpoN*1250::Tn5 (Table 1), because the Tn5 transposon was inserted after the *rpoN* coding region nucleotide number 1249. The location of the Tn5 insertion in *rpoN*1250::Tn5 was determined by Tn5 plasmid rescue and sequencing of the DNA flanking the Tn5 transposon. Briefly, *rpoN*1250::Tn5 genomic DNA was isolated (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA), digested with *Eco*RI (New England Biolabs, Ipswich, MA, USA), circularized with T4 DNA ligase (New England Biolabs) and introduced into Transformax EC100D *pir*-116 electrocompetent *Escherichia coli* (Epicentre) cells. Plasmid DNA was isolated from the resulting kanamycin-resistant colonies (QIAquick Kit, Qiagen, Valencia, CA, USA) and sequenced at the Genomics Core Facility at Pennsylvania State University using sequencing primers provided with the EZ-Tn5<R6K<oriKan-2>Transposome Kit (Epicentre). The position of the flanking DNA sequence in the *E. amylovora* strain CFBP 1430 genome (Smits *et al.*, 2010) was determined using the National Center for Biotechnology Information (NCBI)'s BLASTN program (Altschul *et al.*, 1990).

The *rpoN* gene is known to be essential for virulence in other important plant pathogenic bacteria (Chatterjee *et al.*, 2002; Hutcheson *et al.*, 2001). In *Pseudomonas syringae* pv. *syringae* and *Pectobacterium carotovorum* ssp. *carotovorum*, *rpoN* controls the expression of traits related to virulence, including the T3SS, flagella, alginate production (exopolysaccharide) and pili (Chatterjee *et al.*, 2002; Hutcheson *et al.*, 2001). Furthermore, in *P. syringae* pv. *glycinea* PG4180, *rpoN* controls the production of coronatine, a phytotoxin which functions as a virulence factor in bacterial blight of soybean (Alarcón-Chaidez *et al.*, 2003). It has also been demonstrated that *rpoN* controls flagellar protein expression, swimming and swarming, and antifungal biocontrol activity of *Pseudomonas fluorescens* CHA0 (Péchy-Tarr *et al.*, 2005). Expression of the *E. amylovora rpoN* gene is induced during infection of immature pear fruit tissue (Zhao *et al.*, 2005); however, the possible involvement of *rpoN* in *E. amylovora* pathogenicity has not been tested.

Fig. 1 Scale representation of the *rpoN* genomic neighbourhood, including the location of the Tn5 insertion in mutant *rpoN*1250::Tn5 and the genomic segment used for complementation.

The *E. amylovora rpoN* gene encodes a predicted RNA polymerase σ^{54} factor subunit. Homologous RpoN σ^{54} factors are required for the activation and expression of virulence genes, including *hrp* genes, in plant pathogenic bacteria, such as *Pe. carotovorum* ssp. *carotovorum* (Chatterjee *et al.*, 2002) and *P. syringae* pv. *syringae* (Hutcheson *et al.*, 2001; Xiao *et al.*, 1994). It has also been demonstrated that the RpoN σ^{54} factor is part of a complex that activates the expression of *hrpL* in *P. syringae* pv. *syringae* (Xiao *et al.*, 1994). Based on the sequence similarity of the *hrpL* and *rpoN* genes in *P. syringae* pv. *syringae* and *E. amylovora*, it has been hypothesized that RpoN is also part of a complex that activates the expression of *hrpL* in *E. amylovora* (Vanneste, 2000), which would probably make RpoN an essential virulence regulator in *E. amylovora*. This is in contrast with the *E. amylovora* RpoS alternative σ factor, which is required for environmental stress tolerance, but not for disease processes in apple (Anderson *et al.*, 1998).

Indeed, the *rpoN*1250::Tn5 mutant did not cause fire blight symptoms in mature 'Fuji' trees inoculated by shoot tip wounding (Fig. 2). The *rpoN*1250::Tn5 mutant also did not cause fire blight disease symptoms in immature apple fruits at 7 days post-inoculation (dpi) (Fig. 3a), and *rpoN*1250::Tn5 populations were twelve orders of magnitude lower than in the wild-type in apple fruits at 7 dpi (Fig. 3b). The *rpoN* gene was amplified from wild-

Fig. 2 The *rpoN* gene was required for *Erwinia amylovora* pathogenicity on apple tree shoots. Three-year-old, dormant, bare-rooted 'Fuji' apple trees grafted on ELMA 26 rootstocks were potted and grown in a glasshouse for 7 weeks with regular soluble fertilizer treatments to promote and maintain vigour. The trees were then wounded inoculated on actively growing shoot tips with the indicated *E. amylovora* strains at 1×10^8 colony-forming units (CFU)/mL in 10 mM MgCl₂. Twenty shoots in total were inoculated with each strain; four trees were used for each strain and five shoots were inoculated on each tree. Each tree was inoculated with one strain only. Mock-inoculated shoots were wounded and inoculated with 10 mM MgCl₂. The entire experiment was performed twice, with similar results each time. Inoculations were performed as described previously (Jensen *et al.*, 2003). The extent of shoot necrosis at 7, 14 and 21 days post inoculation (dpi) is shown. The extent of shoot necrosis was calculated by dividing the length of the necrotic portion of the shoot, measuring from the shoot tip, by the total shoot length measured from the shoot tip to the shoot junction with the previous year's wood, and expressed as a percentage. Statistical analyses were completed using PROC GLM in SAS (version 9.2; SAS Institute, Cary, NC, USA), with strain treated as the independent variable and log₁₀ CFU as the dependent variable. *Post-hoc* comparisons were evaluated using Tukey's test, with $\alpha = 0.05$. Error bars represent the standard error of the mean of twenty replicate samples per strain. Bars with the same letters are not statistically significantly different.

type *E. amylovora* HKN06P1 genomic DNA using the forward primer 5'-CAGCGCCTTGCGTCTTGA-3' and reverse primer 5'-GCCGCCGTTTACATGCAGCG-3' and 12 PCR cycles with an annealing temperature of 60 °C. The resulting 1792-bp product was cloned into pCR2.1 (TOPO TA Kit, Invitrogen, Grand Island, NY, USA), creating pCR2.1-*rpoN* (Table 1), and sequenced at the Pennsylvania State University Genomics Core Facility using T7 and M13R as forward and reverse primers, respectively. This cloned segment of *E. amylovora* HKN06P1 DNA was identical in sequence

Fig. 3 The *rpoN* gene was required for *Erwinia amylovora* pathogenicity on immature apple fruits. (a) Immature 'Gala' apple fruit halves at 7 days post-inoculation (dpi) with 2×10^6 colony-forming units (CFU) of the indicated bacterial strains into a wound on the surface of the fruit as described previously (Lee *et al.*, 2010). Tissue necrosis and bacterial ooze are a symptom and a sign, respectively, of *E. amylovora* infection in immature apple fruits. (b) Bacterial populations in immature 'Gala' apple fruit halves at 7 dpi with 2×10^6 CFU of the indicated bacterial strains, determined by serial dilution plating as previously described (Lee *et al.*, 2010). Bars with the same letter are not significantly different according to Tukey's test (log₁₀). The same apples shown in (a) were used for the assay shown in (b). The experiment was repeated three times with at least five fruit halves inoculated per strain. Every experiment repetition had similar results; the results of representative experiments are shown. Error bars represent the standard error of the mean of five replicate samples per strain. Statistical analyses were performed in the same way as described in the legend of Fig. 2.

to that of the *E. amylovora* CFBP 1430 reference genome, and included the entire *rpoN* gene as well as 116 and 49 bp of flanking genes *yhbH* and *yhbG*, respectively (Fig. 1). Plasmids pCR2.1 (empty cloning vector; Table 1) and pCR2.1-*rpoN* were introduced into *rpoN*1250::Tn5 using electroporation, creating *E. amylovora* strains *rpoN*1250::Tn5(pCR2.1) and *rpoN*1250::Tn5(pCR2.1-*rpoN*), respectively (Table 1). Strain *rpoN*1250::Tn5(pCR2.1-*rpoN*)

Fig. 4 The *rpoN* gene was required for the *Erwinia amylovora* hypersensitive response (HR) in tobacco. Tobacco (*Nicotiana tabacum* L. cv. Glurk) leaf sections were infiltrated with suspensions of 1×10^8 colony-forming units (CFU)/mL in 10 mM MgCl_2 of the indicated strains using a syringe without a needle, as described previously (Sinn *et al.*, 2008). The HR was indicated by collapse and death of the infiltrated region. The HR was scored at 24 h post-inoculation (hpi); photographs were taken at 72 hpi for better contrast. The tobacco plants were 6 weeks old. The experiment was repeated three times with at least eight tobacco plants used for each experiment. The experiment had identical results each time; a representative leaf is shown.

caused fire blight symptoms when inoculated onto mature apple tree shoots and immature apple fruit, whereas strain *rpoN1250::Tn5*(pCR2.1) did not (Fig. 2 and Fig. 3a). In addition, strain *rpoN1250::Tn5*(pCR2.1-*rpoN*) population sizes in inoculated apple fruits were indistinguishable from wild-type population sizes at 7 dpi, whereas strain *rpoN1250::Tn5*(pCR2.1) populations were similar to those of *rpoN1250::Tn5* (Fig. 3b). Thus, pCR2.1-*rpoN* complemented the *rpoN1250::Tn5* mutant virulence defect on apple tree shoots and immature apple fruit. This indicates that the *rpoN* gene is specifically required for *E. amylovora* virulence in apple, and confirms that the *rpoN1250::Tn5* virulence defect is caused by the Tn5 transposon insertion in the *rpoN* gene.

Erwinia amylovora elicits an HR when infiltrated into the leaves of nonhost tobacco plants (Giorgi and Scortichini, 2005). The HR is a mechanism used by plants that allows them to prevent the spread of pathogens inside their tissue and is characterized by rapid, local cell death in the infected zone (Morel and Dangl, 1997; Stakman, 1915). The degree of HR elicitation by *E. amylovora* in tobacco plants is correlated with virulence in apple fruits (Lee *et al.*, 2010). To determine whether the *rpoN1250::Tn5* mutant was defective in causing an HR on tobacco, the wild-type and *rpoN1250::Tn5* mutant strains were infiltrated into tobacco leaves. Wild-type *E. amylovora* caused an HR after 24 h, whereas *rpoN1250::Tn5* did not (Fig. 4). Furthermore, *rpoN1250::Tn5*(pCR2.1-*rpoN*) caused an HR, whereas *rpoN1250::Tn5*(pCR2.1) did not (Fig. 4), indicating that plasmid pCR2.1-*rpoN* complemented the *rpoN1250::Tn5* mutant HR defect.

The T3SS-secreted HrpN protein is a critical component of *E. amylovora* virulence in hosts and is required for elicitation of

Fig. 5 The *rpoN* gene was required for HrpN protein expression in cultured *Erwinia amylovora* cells. Protein gel immunoblots detecting HrpN protein in cell pellets and cell-free culture supernatants are shown. The indicated strains were grown under *hrp*-inducing conditions and proteins from cell pellets and cell-free culture supernatant fractions were subjected to immunoblot analysis to detect the presence of HrpN protein as described previously (Sinn *et al.*, 2008). The entire experiment was conducted twice with identical results.

the HR in tobacco (Wei *et al.*, 1992). A defect in HrpN expression or secretion might contribute to the pathogenicity and HR defects of *rpoN1250::Tn5*. Therefore, we tested whether HrpN was expressed and secreted by *rpoN1250::Tn5* cells growing in *hrp*-inducing culture medium (Huynh *et al.*, 1989; Sinn *et al.*, 2008). Protein samples from *E. amylovora* cell pellet and cell-free culture supernatant fractions were prepared as described previously (Lee *et al.*, 2010; Sinn *et al.*, 2008). The proteins were resolved by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and detected with a polyclonal anti-HrpN antibody (gift of S. V. Beer, Cornell University, Ithaca, NY, USA; Wei *et al.*, 1992). As expected, HrpN protein was detected in wild-type cells and culture supernatant; in contrast, HrpN was not detected in cells or supernatant of *rpoN1250::Tn5* (Fig. 5). Furthermore, HrpN was detected in *rpoN1250::Tn5*(pCR2.1-*rpoN*) cells and supernatant, whereas HrpN was not detected in *rpoN1250::Tn5*(pCR2.1) cells and supernatant (Fig. 5). These results indicate that *rpoN1250::Tn5* mutant cells did not express HrpN protein in *hrp*-inducing medium, and that pCR2.1-*rpoN* restored HrpN expression and secretion by *rpoN1250::Tn5* cells. A *hrcC118::Tn5* T3SS mutant (Table 1) showed detectable HrpN in the cells, but not the supernatant (Fig. 5), as expected (Wei *et al.*, 2000). These results indicate that a lack of HrpN expression probably contributed to the pathogenicity and HR defects of the *rpoN1250::Tn5* mutant.

In order to determine whether basic metabolic processes were intact in *rpoN1250::Tn5*, the ability of the *rpoN1250::Tn5* mutant to grow on minimal medium was compared with that of the wild-type. The *rpoN1250::Tn5* mutant grew as well as the wild-type on solid M9 minimal medium (MM) (Sambrook *et al.*, 1989) with 0.4% sorbitol (Fig. 6a) or 0.4% glucose (Fig. S1a, see Supporting Information). Furthermore, the *rpoN1250::Tn5* mutant

Fig. 6 The *rpoN1250::Tn5* mutant was not auxotrophic. (a) Growth of the indicated strains streaked onto M9 minimal medium (MM) agar with 0.4% sorbitol after 48 h at 28 °C. (b) Growth of the indicated strains in liquid MM with 0.4% sorbitol after 48 h of shaking at 150 rpm at 28 °C. The auxotrophic strain had a Tn5 insertion in the *porF* gene, which is predicted to encode an amidophosphoribosyltransferase. There was no significant difference between the wild-type and *rpoN1250::Tn5* at 0 or 48 h, based on Tukey's test (log₁₀). Experiments were repeated three times with similar results each time; results of representative experiments are shown. Error bars represent the standard error of the mean of three replicate samples. Statistical analyses were performed in the same way as described in the legend of Fig. 2. CFU, colony-forming units.

grew as well as the wild-type in liquid MM with 0.4% sorbitol (Fig. 6b) or liquid MM with 0.4% glucose (Fig. S1b). These results indicate that *rpoN1250::Tn5* is a prototroph. This contrasts with *P. syringae* pv. *glycinea* and *P. fluorescens* *rpoN* mutants, which did not grow in M9 minimal medium unless supplemented with glutamate, glutamine or aspartate (Alarcón-Chaidez *et al.*, 2003; Péchy-Tarr *et al.*, 2005).

Our results indicate that *rpoN* is essential for *E. amylovora* to cause fire blight symptoms in apple tree shoots and to grow and cause fire blight symptoms in detached immature apple fruits. Our results also indicate that *rpoN* is essential for the *E. amylovora*-induced HR in nonhost tobacco plant leaves. The results also show that the *E. amylovora* *rpoN* gene is required for the production of HrpN protein in *hrp*-inducing medium. The lack of HrpN protein production by *rpoN1250::Tn5* probably contributes to the lack of virulence of *rpoN1250::Tn5* on apple fruits and shoots and the inability of *rpoN1250::Tn5* to trigger an HR in tobacco. Complementation of the *rpoN1250::Tn5* mutant phenotype with pCR2.1-*rpoN* indicates that pCR2.1-*rpoN* contained a functional copy of the *rpoN* gene. As the *rpoN1250::Tn5* mutant was not an auxotroph, its virulence and avirulence defects are probably not caused by the disruption of a basic metabolic process that might affect its ability to grow in the host. Instead, our results indicate that *rpoN* plays a specific role in *E. amylovora* virulence. The pathogenicity defect of the *rpoN1250::Tn5* mutant and its lack of HrpN expression are consistent with RpoN being a key virulence system expression regulator in *E. amylovora*, just as it is in *P. syringae* and *Pe. carotovorum*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 The *rpoN1250::Tn5* mutant was not auxotrophic. (a) Growth of indicated strains streaked onto M9 minimal medium (MM) agar with 0.4% glucose after 48 h of growth at 28 °C. (b) Growth of indicated strains in liquid MM with 0.4% glucose after 48 h of shaking at 150 rpm at 28 °C. The auxotrophic strain used was another Tn5 mutation in the *porF* gene. There was no significant difference between the wild-type and *rpoN1250::Tn5* at 0 or 48 h, based on Tukey's test ($\log_{10}$). Statistical analyses were completed using PROC GLM in SAS (version 9.2; SAS Institute, Cary, NC, USA), with strain treated as the independent variable and $\log_{10}$ colony-forming units (CFU) as the dependent variable. *Post-hoc* comparisons were evaluated using Tukey's test, with $\alpha = 0.05$. Error bars represent the standard error of the mean of three replicate samples per strain. Experiments were repeated three times with similar results each time; results of representative experiments are shown.