Expression of nip<sub>P,w</sub> of Pectobacterium wasabiae is dependent on functional flgKL flagellar genes

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Abstract

While flagellum-driven motility is hypothesized to play a role in the virulence of *Pectobacterium* species, there is no direct evidence that genes involved in flagellum assembly regulate the synthesis of virulence factors. The purpose of this study was to identify genes that affect the production or secretion of necrosis-inducing protein (Nip) in the strain SCC3193. Transposon mutagenesis of an RpoS strain overexpressing Nip\textsubscript{P.w} was performed, and a mutant associated with decreased necrosis of tobacco leaves was detected. The mutant contained a transposon in the regulatory region upstream of the flagellar genes *flgK* and *flgL*. Additional mutants were generated related to the flagellar genes *fliC* and *fliA*. The mutation in *flgKL*, but not those in *fliC* and *fliA*, inhibited *nip\textsubscript{P.w}* transcription. Moreover, the regulatory effect of the *flgKL* mutation on *nip\textsubscript{P.w}* transcription was partially dependent on the Rcs phosphorelay. Secretion of Nip\textsubscript{P.w} was also dependent on a type II secretion mechanism. Overall, the results of this study indicate that the *flgKL* mutation is responsible for reduced motility and lower levels of *nip\textsubscript{P.w}* expression.
Introduction

Several *Pectobacterium* species cause soft-rot and blackleg in potatoes (Pérombelon, 2002). Soft-rot symptoms are mainly caused by the production of plant cell wall-degrading enzymes (PCWDE), which are acting as potent virulence factors (Toth & Birch, 2005). This study deals with a well-characterized *Pectobacterium* strain SCC3193, that was recently reclassified as *Pectobacterium wasabiae* (P.w). In P.w, motility and PCWDE production traits are coupled to, and indispensable for, successful infection of the host plant (Cui et al., 2008; Andresen et al., 2010). Expression of virulence in P.w is modulated at transcriptional (Andresen et al., 2007; Cui et al., 2001; Hyytiäinen et al., 2001, 2003; Mukherjee et al., 2001) and post-transcriptional (Chatterjee et al., 1995, 2009; Cui et al., 2008; Kõiv et al., 2013) levels, but is also regulated by quorum sensing (for a review, see Põllumaa et al., 2012). PCWDE should be secreted to reach their target substrates in plant cell wall. For that, a type II secretion system, also known as the Out-system, is used in case of pectinases and the cellulase (Reeves et al., 1994), whereas the type I Prt machinery is applied for the secretion of protease (Marits et al., 1999).

In addition to PCWDE, many plant pathogenic bacteria exploit certain proteins as toxins to suppress plant defence responses (Espinosa & Alfano, 2004; Friesen et al., 2008). In P.w, a toxin-like necrosis-inducing protein (Nip$_{P.w}$) was isolated and shown essential for different stages of plant-bacterial interactions (Mattinen et al., 2004; Pemberton et al., 2005). When the nip$_{P.w}$ gene was inactivated in P.w, symptoms of soft-rot disease in potato tubers were significantly reduced (Mattinen et al., 2004). Importantly, the deduced sequence of Nip$_{P.w}$ showed significant homology to necrosis- and ethylene-inducing (Nep1) protein of fungi and oomycetes (Mattinen et al., 2004). The effect of nip mutation on virulence was also obvious in case of *Pectobacterium atrosepticum* (*P. atrosepticum*), where the nip mutation reduced both potato soft rot and stem rotting (Pemberton et al., 2005). In accordance with suggested toxin-like function of Nep1-like proteins (NLPs), including the Nip$_{P.w}$, Ottmann et al. (2009) demonstrated that they accelerate disease progression via disintegration of plant cell plasma membranes.

Though the NLPs are probably playing important role in pathogenesis, only few data are
available on their regulation in bacterial pathogens. In *P. atrosepticum* the *nip* was proposed to be regulated by the LuxR family regulator EccR, and the global regulator RsmA (Pemberton *et al*., 2005). In *P.w* strain SCC3193, expression of *nipP.w* was reported to be negatively controlled by RpoS (Mattinen *et al*., 2004).

Alongside with production of certain proteins (e.g. PCWDE and toxic proteins), flagellar motility is an important virulence determinant in several pathogenic bacteria (Gauger *et al*., 2007; Matsumoto *et al*., 2003; Hossain *et al*., 2005). Intriguingly, in *P.w*, motility and PCWDE production are coregulated: the master regulator FlhDC of flagellar genes is also implicated in regulation of PCWDE production (Cui *et al*., 2008; Chatterjee *et al*., 2009). In addition, we have previously demonstrated that both PCWDE synthesis and motility are regulated by the Rcs phosphorelay (Andresen *et al*., 2007). The Rcs phosphorelay negatively affects PCWDE expression by inhibiting the expression of *flhDC* and *rsmB* (Andresen *et al*., 2010). In this phosphorelay, the RcsC sensor kinase is autophosphorylated in response to environmental stimuli and activates the response regulator RcsB by phosphoryl group transfer via RcsD, a phosphotransmitter protein.

Within flagella regulons of *Salmonella* and *E. coli*, the operons comprise three classes. Class I genes, the *flhD* and *flhC*, represent the master regulatory operon responsible for the control over flagellar genes expression (for a review, see Prüβ, 2000; Wang *et al*., 2006). The FlhD and FlhC proteins activate class II genes, which participate in formation of the hook basal body (HBB) structure, synthesis of the sigma factor FliA (σ²₈) and the anti-sigma factor FlgM (Liu & Matsumura, 1994). Transcription of class III genes requires the FliA protein, and the products of these genes comprise the flagellar filament and the chemosensory machinery (Karlinsey *et al*., 2000).

Given that flagellar mutants of human pathogens have pleiotropic phenotype, it has been hypothesized that genes involved in flagellum assembly play also a role in production and/or secretion of virulence factors, thereby they influence bacterial-host interactions independently of the motility trait (Ghelardi *et al*., 2002; Konkel *et al*., 2004; Bouillaut *et al*., 2005). In present study, we identify novel mutations that affect expression of the necrosis-inducing protein of *P. wasabiae* (Nip*P.w*) and will show that the *flgKL* mutation causes both reduced motility and lower levels of *nipP.w* expression.
Materials and methods

Bacterial strains, vectors, transduction, and growth conditions

The strains and plasmids used in this study are listed in Table 1. *P. w* and *Escherichia coli* strains were grown at 30°C and 37°C, respectively. They were grown on LB medium or M9 minimal salts medium containing 10% potato extract (v/v) and appropriate trace elements (Miller, 1972; Sambrook & Russell, 2001). When required, antibiotics were added as follows: ampicillin (Amp) 150 µg ml⁻¹, kanamycin (Km) 100 µg ml⁻¹, and chloramphenicol (Cm) 25 µg ml⁻¹. To induce the hypersensitive response (HR) in tobacco plants, *P. w* cells were grown for three days at 15°C on solid minimal induction medium (Huynh *et al.* 1989) supplemented with 10 mM mannitol and 0.5% sucrose (w/v). *Nicotiana tabacum* cv. Samsun plants used in the necrosis assays were grown in greenhouses at approximately 22°C. Plants were used before flowering.

Chromosomal markers were transduced with T4GT phage as previously described (Pirhonen *et al.*, 1991).

DNA manipulations

Standard DNA techniques described in Sambrook and Russell (2001) were applied. The oligonucleotide primers used for PCR amplifications are listed in Table 2.

Transposon mutagenesis and construction of mutant strains

Transposon mutagenesis of the *rpoS* strain SCC8003 was performed using λ phage 1105 as previously described (Pirhonen *et al.*, 1991). To localise the inserted transposon in motility mutants, arbitrary PCR primers (Caetano-Annoles, 1993) were used in combination with Tn10-specific primers (listed in Table 2) to amplify flanking DNA sequences. Mutated genes were identified and the inserts were localised by sequencing the amplified fragments.
The fliA::Cm, fliC::Cm, and outC::Cm fragments were amplified using the primers FliAP1 and FliAP2, FliCP1 and FliCP2, OutCP1 and OutCP2 respectively (listed in Table 2), with plasmid pKD3 as template. Chromosomal genes were replaced with mutated genes using the λ Red system (Datsenko & Wanner, 2000).

**Enzyme assays**

For β-glucuronidase (GusA) assays, cells were grown at 28°C on solid M9 minimal medium supplemented with 10% potato extract (v/v). GusA activity was measured 24 h after inoculation using p-nitrophenyl β-D-glucuronide as substrate (Novel et al., 1974). The degradation product, p-nitrophenol (pNP), was detected at 405 nm, and specific GusA activity was expressed as nmol pNP liberated min⁻¹ (OD₆₀₀ unit)⁻¹.

**Motility assay**

Motility was evaluated on LB soft-agar plates (0.3% (w/v) agar). A sample (1 ml) of overnight culture of each strain was used to make a dilution in M9 to OD₆₀₀=2.0. Cultures were stabbed into the centre of soft-agar plates using a sterile inoculation needle. Plates were incubated at 30°C for 24 h.

**Western blotting**

Cultures of P.w for Western blotting were grown at 30°C in LB medium to an OD₆₀₀ of 1.0 before induction with 0.1M IPTG. After 4 h induction, the cells were harvested by centrifugation at 4°C and extracellular proteins were precipitated overnight at 4°C with 5% (v/v) trichloroacetic acid (final concentration). The precipitates were isolated by centrifugation at 10,000g for 20 min at 4°C and the pellets were washed twice with ice-cold acetone, air-dried, and then resolubilized in 0.1M sodium phosphate buffer, pH 7.0. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad). Equal amounts of total protein (10 μg) were loaded on 12.5% SDS-polyacrylamide (w/v) gels and transferred to NitroBind nitrocellulose membranes (Hybond™-ECL™, Amersham Biosciences UK Limited). For Western blotting, the membranes were probed with a 1:2500 dilution of monoclonal antibodies raised against
Nip\textsubscript{P,w} in rabbits (LabAs Ltd., Tartu, Estonia) followed by treatment with goat anti-rabbit antibodies conjugated with alkaline phosphatase (Promega Biotec). The blots were developed using bromochloroindolyl phosphate/nitroblue tetrazolium.

**Results**

1. **Generation of *P. wasabiae* flagellar mutants**

To identify gene(s) affecting nip\textsubscript{P,w} expression, an rpoS mutant, SCC8003, which produces elevated levels of Nip\textsubscript{P,w} (Mattinen et al., 2004), was subjected to transposon mutagenesis. For these studies, a total of 500 mutant colonies were grown individually and inoculated into tobacco leaf tissue. Only one mutant was associated with an absence of tissue necrosis on tobacco leaves (Fig. 1), and sequencing revealed a TnKm insertion in the intergenic region upstream of two flagellar genes, flg\textit{K} and flg\textit{L}. To characterize the effect of this flg\textit{KL} mutation on nip\textsubscript{P,w} expression, a transcriptional fusion unit of nip\textsubscript{P,w} and gus\textit{A} (pKT3.1) (nip\textsubscript{P,w}::gus\textit{A}) was introduced into this rpo\textit{S}/flg\textit{KL} double mutant. Lower β-glucuronidase activity was produced by this double mutant than by the rpo\textit{S} single mutant (Fig. 2). Furthermore, when the flg\textit{KL} mutant was transduced into a wild type background using T4GT, the activity of the nip\textsubscript{P,w}::gus\textit{A} transcriptional fusion was lower in the flg\textit{KL} mutant than in the wild type strain. The phenotypic responses elicited by expression of flg\textit{KL} from a low-copy plasmid (pLK) restored nip\textsubscript{P,w}::gus\textit{A} (Fig. 2) expression and motility in an flg\textit{KL}negative strain (Fig. 3).

To test whether mutations in other flagellar genes affect nip\textsubscript{P,w} expression, insertion mutants were generated in genes encoding the putative filament protein, flagellin (fli\textit{C}), and in sigma factor σ\textsuperscript{28} (fli\textit{A}). All of these mutants were non-motile on 0.3% agar plates (Fig. 3).

2. **Nip\textsubscript{P,w}::gus\textit{A} expression in flagellar mutants**

To characterize the influence of other flagella genes, the nip\textsubscript{P,w}::gus\textit{A} fusion was introduced into the flg\textit{KL}, fli\textit{C}, fli\textit{A}, and flh\textit{D} mutants. As in the flg\textit{KL} mutant, expression
of the nip<sub>P,w</sub>:::gusA fusion was 5-6-fold lower in the flhD mutant than in the wild type
strain (Fig. 4). In the fliA and fliC mutants, expression of nip<sub>P,w</sub>:::gusA was comparable to
the level of expression in the wild type strain (Fig. 4). These results suggest that
transcription of nip<sub>P,w</sub> is not under the control of sigma factor σ<sup>28</sup> in <i>P. wasabiae</i>, and that
functional fliA, and fliC genes important for motility are not involved in regulating nip<sub>P,w</sub>
transcription.

3. Involvement of the Rcs system in the regulation of nip<sub>P,w</sub>

To test the hypothesis that an flgKL mutation affects nip<sub>P,w</sub> expression via the Rcs
system, expression of nip<sub>P,w</sub>:::gusA was measured in a wild type strain, as well as in single
mutants (e.g., rcsB, rcsD, rcsC, and rcsF) and the double mutant (rcsBflgKL). While
inactivation of rcsF had no effect on nip<sub>P,w</sub>:::gusA expression, nip<sub>P,w</sub>:::gusA expression
was twice as great in the rcsB, rcsD, and rcsC mutants as in the wild type strain (Fig. 5).
Furthermore, compared to rcsB mutants, the double mutant did not restore expression of
the nip<sub>P,w</sub>:::gusA fusion. These results suggest that the negative effect of flgKL mutation
on nip<sub>P,w</sub> expression is only partly mediated through the Rcs system.

4. Secretion of Nip<sub>P,w</sub> protein in the wild type strain versus different mutants

To test the hypothesis that mutations in flagellar genes affect Nip<sub>P,w</sub> secretion, the
export of Nip<sub>P,w</sub> expressed ectopically in fliA, fliC, flhD and flgKL flagellar mutants was
examined. For these analyses, nip<sub>P,w</sub> expression was placed under the control of a plac
promoter (ρNip<sub>P,w</sub>) in order to be independent of mechanisms affecting nip<sub>P,w</sub>
transcription, thus enabling the effects on Nip<sub>P,w</sub> secretion to be examined directly.
To determine the involvement of the flagellar secretion apparatus in Nip<sub>P,w</sub>
secretion, the secretion of Nip<sub>P,w</sub> was examined in the fliA, fliC, flhD, and flgKL mutants.
Using immunoblotting, culture supernatants from the fliA, fliC, flhD, and flgKL mutants
were analyzed. In all the samples studied the amount of Nip<sub>P,w</sub> in the supernatant was
comparable to that from the wild type strain (Fig. 6). Taken together, these results
suggest that Nip<sub>P,w</sub> is unlikely to be secreted through the flagellar type III secretion
system in <i>P. wasabiae</i>.
In parallel, Nip<sub>P.w</sub> protein secretion was assayed in the outC- mutant. As illustrated in Figure 6, immunoblotting revealed almost undetectable levels of Nip<sub>P.w</sub> in the supernatant of the outC- mutant. In combination, these results suggest that a functional type II (Out-system) secretion system is required for Nip<sub>P.w</sub> secretion.

Discussion

When an rpoS strain overexpressing Nip<sub>P.w</sub> was subjected to mutagenesis, a mutant exhibiting reduced necrosis in tobacco tissue was identified. The relevant mutation was localized to the intergenic region upstream of two flagellar genes, flgK and flgL, suggesting that flagella are important for the production of Nip<sub>P.w</sub>. To characterize the mechanism involved in the phenotype of the mutant further, several motility mutants were analyzed.

The transposon insert caused a clear decrease in nip<sub>P.w</sub> transcription in flgKL (Fig. 2) but had no effect on the expression of PCWDE genes (data not shown). Without assembly of the FlgKL proteins, the HBB complex is most likely not functional and filament subunits are unable to polymerize. However, rather than a direct effect on nip<sub>P.w</sub> transcription, it is hypothesized that inefficient assembly of the flagellar HBB complex or mislocation of the FliC monomers due to the defective HBB complex leads to activation of the Rcs phosphorelay system and decreased nip<sub>P.w</sub> transcription. The Rcs phosphorelay system has previously been identified as a repressor of genes responsible for the PCWDE synthesis (Andresen et al., 2007, 2010). The higher expression of the nip<sub>P.w</sub>::gusA fusion in the rcsBflgKL double mutant than in the flgKL mutant (Fig. 5) also suggests that a mutation in flgKL affects nip<sub>P.w</sub> transcription by modulating the activity of the Rcs phosphorelay system. The Rcs phosphorelay responds to a wide range of conditions, including structural perturbations in the peptidoglycan layer or cell membrane (Clarke, 2010). Our findings are also consistent with the observation that artificial membrane stress induced by deletion of the cytoplasmic membrane-anchored DjlA and outer membrane OmpG protein activates the Rcs phosphorelay system in different bacteria (Chen et al., 2001; Shiba et al., 2006). In addition, it has been demonstrated that the
negative effects of the Rcs phosphorelay system on PCWDE in *P. wasabiae* are achieved through the Rsm system. For example, RcsB can repress *rsmB* expression directly by binding the *rsmB* promoter, or indirectly by reducing *flhDC* transcription and increasing RsmA levels in the cell (Andresen *et al.*, 2010). These data are also consistent with the work of Pemberton and associates (2005), who observed a decrease in *nip* expression in the absence of homoserine lactone production, which resulted in increased levels of RsmA. On the other hand, increased production of Nip in the *rpoS* mutant could be caused by reduced levels of RsmA (Mattinen *et al.*, 2004). Therefore, it remains unclear whether RsmA affects the stability of *nip* transcripts directly, or indirectly. The results of the present study, where expression of the *nip*:gus fusion in the *rcsB* double negative mutant was lower than in the wild type strain, suggest that mutation in flagellar genes is likely to trigger an additional Rcs phosphorelay-independent pathway to repress *nip* transcription. It is also possible that defects in HBB assembly activate an unknown regulatory system, which in turn activates the Rsm system. A possible mechanism leading to this phenotype could be feedback regulation of *flhDC* due to defective flagellar assembly. However, there was no difference in *flhDC* expression between the *flgKL* mutant and the wild type strain, suggesting that feedback regulation is not a likely explanation.

Recently, Cui *et al.* (2008) described a complex interplay between motility and bacterial virulence, where the master regulator of flagellar genes, FlhDC, concomitantly controls flagella and PCWDE synthesis. Correspondingly, the results of the present study also demonstrate that FlhDC is necessary for *nip* expression (Fig. 4). Furthermore, the present results also demonstrate that mutation in a gene involved in the assembly of HBB cause reduction in motility as well as decreases *nip* expression.

Mutations in *fliA* (genes involved in the regulation of class III flagellar genes), and *fliC* (a class III structural flagellar gene), resulted in non-motile cells (Fig. 3) in which *nip* expression was unchanged (Fig. 4). These results suggest that the *fliA*, and *fliC* mutants still produce a normal HBB, yet flagellar proteins do not accumulate inside the cell or in the periplasm. In addition, the cell membrane remains intact, thereby not activating the Rcs phosphorelay system and inhibiting *nip* expression.
It has previously been demonstrated that the bacterial flagellar export apparatus also functions as a secretion system for non-flagellar proteins (Young et al., 1999; Ghelardi et al., 2002). This ability of the flagellar export machinery to maintain two types of function supports the hypothesis that this system also contributes to Nip<sub>P,w</sub> secretion. However, the detection of similar levels of Nip<sub>P,w</sub> in immunoblots of supernatant samples from all flagellar mutants harboring the *nip*<sub>P,w</sub> gene under the control of the lac-promoter indicates that compromise of the flagellar export machinery (especially in *flgKL* mutant) does not abolish Nip<sub>P,w</sub> export. Nip<sub>P,w</sub> contains a Sec-dependent signal sequence, which allows it to be secreted through a type II secretion system. Coulthurst and associates (2008) also observed that Nip was secreted in an Out-dependent manner in *Pectobacterium atrosepticum*. Taken together, the results of the present study and those of previous studies indicate that Nip<sub>P,w</sub> is transported out of the cell via a type II secretion system (as are many other known virulence factors, e.g. cellulases and pectinases) in *P. wasabiae* (Reeves et al., 1994; Corbett et al., 2005).

In summary, the present study provides evidence suggesting that the reduced virulence of flagella mutants may not be solely due to reduced motility, but also to reduced production of one or more virulence factors. In addition, although the mechanistic details of the effect of the *flgKL* mutation on *nip*<sub>P,w</sub> gene transcription were not completely identified, at least part of this pathway was found to involve the Rcs phosphorelay system.

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References


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### Table 1. Strains and plasmids used in this study

<table>
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<th>Strains</th>
<th>Description</th>
<th>Source orReference</th>
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<td><em>hsdS gal</em> (<em>λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1</em>)</td>
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<td>DH5-α</td>
<td><em>supE4, ΔlacU169, (lacZA15)</em>, <em>hasdR17, recA1, endA1, gyrA 96, thi-1, relA1</em></td>
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<td><strong>P. wasabiae</strong></td>
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<td>Wild type strain</td>
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<td>Marits <em>et al.</em>, 2002</td>
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<td>pKT3.1</td>
<td>Vector pMW119::<em>gusA</em> containing the promotor region of <em>nip&lt;sub&gt;p,w&lt;/sub&gt;</em> amplified from wt SCC3193</td>
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<td>Rita Hõrak, unpublished</td>
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<td>pLK</td>
<td>plasmid pLK-Gm containing the Cm&lt;sup&gt;R&lt;/sup&gt; gene amplified from the plasmid pKD3 using primers CmP1 and CmP2 between <em>BglII</em> and <em>NcoI</em> sites (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>The present study</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>opposite direction to the <em>tac</em> promoter; (Gm&lt;sup&gt;R&lt;/sup&gt;)</td>
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Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)*</th>
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<tr>
<td>FliCP1</td>
<td>TATCAACAGTGCCAAAAGACGATGCTGCGG5GCGGAGGC TATCGCTAAACCCTAGCTGGAGCTGCTTTC</td>
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<td>FliCP2</td>
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<tr>
<td>FliAP1</td>
<td>GCGCTATGTTCCACTAGTGCGCCATGAAAGCCTTTCG TTTACAGGTTGTGAGGCTGAGGCTGCTT</td>
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<tr>
<td>FliAP2</td>
<td>ACGGCATAAGTGGTAACGCGCTACCTTGGAACGA ATCATAGCGTTCGTCATGATAATATCCCTCCTT</td>
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<td>OutCP1</td>
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<td>OutCP2</td>
<td>CGTAGCGGGCTTTTCTTTTCGCTGGGCAAGGC5CGTAGTAAC AGACACGCAACCATATGAATATCCCTCCTT</td>
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<tr>
<td>NipP1</td>
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<tr>
<td>CmP2</td>
<td>CGAAATGAGAGCGGTTGATCGG</td>
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</table>

*Sites designed for restriction enzymes are underlined.
**Figure legends**

**Fig. 1.** Induction of cell death in tobacco tissue by *P. w* wt, rpoS mutant, *flgKL* mutant and buffer control. The wt strain and the mutants were grown for three days at 15°C on solid minimal induction medium (Huynh et al., 1989) supplemented with 10 mM mannitol and 0.5% sucrose. *Nicotiana tabacum cv.* Samsun plants used in the HR assays were grown in greenhouses at approximately 22°C. Leaves were photographed two days after infiltration.

**Fig. 2.** Expression of the *nip*::*gusA* fusion in wild type and mutant strains. (A) GUS activity associated with *rpoS*, *flgKL-rpoS*, *flgKL* and *flgKL* (pLK) mutants compared to wild type. Strains were grown on minimal agar plates supplemented with 0.4% glycerol and 10% potato extract at 28°C. Levels of β-glucuronidase (GUS) activity were measured 24 h after inoculation. Bars represent the average from three independent experiments, with standard deviations shown.

**Fig. 3.** Motility of wt strain and flagellar mutants *flgKL*, *fliA*, *flhDC*, *flIC*, *flgKL* (pLK) *flgKL* (pKL) was tested on 0.3% agar. Picture was taken 24 h after inoculation.

**Fig. 4.** GUS activity detected in *flgKL*, *fliA*, *flhDC*, *flIC*, and *outC*-mutants compared to wild type. Strains were grown on minimal agar plates supplemented with 0.4% glycerol and 10% potato extract at 28°C. Levels of β-glucuronidase (GUS) activity were measured 24 h after inoculation. Bars represent the average from three independent experiments, with standard deviations shown.

**Fig. 5.** *Nip::gusA* expression in the *flgKL* mutant is dependent on *rcsB*, *rcsD*, and *rcsC*, but not *rcsF*. The mutant strains assayed are indicated below the graphs, and were grown on minimal agar plates supplemented with 0.4% glycerol and 10% potato extract at 28°C. Levels of β-glucuronidase (GUS) activity were measured 24 h after inoculation. Bars
represents the average from three independent experiments, with standard deviations shown.

**Fig. 6.** Immunoblotting of Nip<sub>P,w</sub> in flagella and *outC* negative mutants. Levels of Nip<sub>P,w</sub> detected in culture supernatants of wt pNip<sub>P,w</sub> (lane 1), *flgKL*/pNip<sub>P,w</sub> (lane 2), control (lane 3), *fliC*/pNip<sub>P,w</sub>, (lane 4), *flgDC*/pNip<sub>P,w</sub> (lane 5), and *fliA*/pNip<sub>P,w</sub> (lane 6). Levels of Nip<sub>P,w</sub> in supernatants of wt/pNip<sub>P,w</sub> (lane 7), control (lane 8), *flgKL*/pNip<sub>P,w</sub> (lane 9), and *outC*/pNip<sub>P,w</sub> (lane 10) strains. Protein extracts were collected from strains grown in LB medium with 0.1 M IPTG, then separated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with an anti-Nip<sub>P,w</sub> antibody.
Figure 1.
Figure 2.

GUS activity (Units)
Figure 3.
Figure 5.

Bar chart showing GUS activity (Units) for various conditions: wt, rcaB, rcaD, rcaC, rcaH, rcaH, rcaB/rcaH.
Figure 6.