

# **BIOLOGY OF *BRENNERIA RUBRIFACIENS*: SCREENING FOR GENES INVOLVED IN PATHOGENESIS**

Ali E. McClean and Daniel A. Kluepfel

## **ABSTRACT**

Deep bark canker of walnut trees is caused by the bacterium, *Brenneria rubrifaciens*. The disease is characterized by a chronic and debilitating decrease in yield and tree vigor. Symptoms of deep bark canker more often appear in trees at least 10 years old. The symptoms include the development of deep longitudinal cracks in trunks, scaffolds, and larger branches which exude a dark colored mixture of bacteria laden sap. Previous research suggests that *B. rubrifaciens* may reside in the vascular tissue of trees where it can lay dormant until a change in environmental conditions, such as water stress occurs resulting in the emergence of virulent bacteria and development of disease. A collection of *B. rubrifaciens* transposon mutants, was subjected to two biological screens in a bid to identify genes required for pathogenicity. Eighty-four mutants were screened in a tobacco leaf bioassay to evaluate hypersensitive response (HR) elicitation. Only 3 of the 84 mutants failed to elicit a HR in tobacco leaves. Genetic analysis revealed these three mutants had insertions in two genetic loci, open reading frames (ORFs) encoding an autoinducer synthetase homolog (mutants Br-212 and Br-512) and a *rpoN*-like  $\sigma_{54}$  factor for RNA II polymerase (mutant Br-415). Twelve mutants were screened for a response on tissue cultured Hartley walnut plants. Two of the twelve mutants tested were reduced in their ability to cause necrosis on tissue cultured walnut plants (mutants Br-212 and Br-415). These findings indicate that two mechanisms:  $\sigma_{54}$  dependent transcription and quorum sensing, a method of communication among bacterial cells, are involved in conferring pathogenesis on walnuts plants and a HR in tobacco.

## **OBJECTIVES**

Identify genes involved in virulence on walnut in order to find novel targets to exploit for deep bark canker control.

## **PROCEDURES**

*Transposon mutagenesis, DNA sequence analysis, and pigment screen* *B. rubrifaciens* 6D370 transposon mutants were prepared and screened for rubrifacine (pigment) production as described previously (McClean, A. E., et al. 2006). DNA sequence analyses of host DNA adjacent to the transposon insertion site were performed.

### **HR bioassays**

*Nicotiana benthamiana* tobacco plants were grown in a greenhouse from seed in autoclaved potted soil. Plants with expanded leaves were transferred to the lab for infiltration experiments. Frozen glycerol stocks (-80°C storage) of wildtype *B. rubrifaciens* and mutants were used to inoculate 10 ml of M9 minimal medium amended with 0.4% w/v glucose. The cultures were grown for 3 days at 28°C 200 rpm in 50 ml corning tubes, sub-cultured 1:10 in 10 ml of fresh

M9, and then incubated overnight under the same conditions. The cultures were centrifuged at 12,000 x g for 5 minutes at room temperature. The supernatant was discarded and the pellets were washed with 10 ml of sterile water and centrifuged again. The bacterial pellets were resuspended in 2 ml of sterile water and adjusted to 1.0 OD 600 units in water using a Beckman Coulter spectrophotometer. The suspensions were vacuum infiltrated into tobacco leaves using a sterile 1 ml syringe. The infiltrated (water soaked) areas were circled with a sharpie marker. The plants were kept in the lab under a light level of approximately 380 lux, 40% relative humidity, and a temperature of 24°C. Results were scored at 24 and 36 hours.

### **Virulence Bioassay**

Bacterial colonies used for walnut virulence assays were streaked from frozen stocks onto YDCA plates and grown for 4 days at 28°C. Walnut plants (cv Hartley) were transferred into individual magenta boxes (1 plant per magenta box) on walnut tissue culture media. All plants were wounded using a sterile scalpel (size 25). Mock treated plants were wounded with the scalpel only. Plants inoculated with *B. rubrifaciens* mutants and wildtype were wounded with a scalpel covered with bacteria scrapped from a culture grown on YDCA. Negative control plants were not wounded. All plants were incubated in a Conviron growth chamber for 30-36 days at 25°C 40% humidity 16 hour light 8 hour dark regimen with a maximum light level of 1525 lux.

## **RESULTS AND DISCUSSION**

Tn5 *B. rubrifaciens* mutants were screened previously for production of rubrifacine (Feistner, G. and Budzikiewicz, H. 1985), the distinctive red pigment produced by *B. rubrifaciens* (McClellan, A. E., et al. 2006) and divided into two groups: 22 pigment minus mutants (pig<sup>-</sup>) and the remainder were pigment producing mutants (pig<sup>+</sup>). The ability of bacterial phytopathogens to cause a HR in the leaves of tobacco plants has been correlated with virulence. Both pig<sup>-</sup> and pig<sup>+</sup> mutants were infiltrated into *N. benthamiana* tobacco leaves to assess their ability to elicit a HR. Of the 8 pig<sup>-</sup> mutants tested only Br-212 and Br-512 were unable to trigger a HR in tobacco. One of the 76 pig<sup>+</sup> Tn5 mutants examined, Br-415 was defective in triggering a HR in tobacco leaves. Hartley cv. walnut plants grown in tissue culture all inoculated with 12 different pig<sup>-</sup> and several pig<sup>+</sup> mutants (Figure 3). Br-212 and Br-415 showed reduced necrosis relative to wildtype *B. rubrifaciens* 6D370.

The open reading frame disrupted by transposon insertion in mutants Br-212 and Br-512 exhibited a high degree of homology at the nucleotide level to the autoinducer synthase gene, *echI*, from *Erwinia chrysanthemi* (Table 1). The *echI* gene is a homolog to the *luxI* gene first described in *Vibrio* species which have been shown to encode proteins involved in cell to cell communication and quorum sensing (Gera, C. and Srivastava, S. 2006; Waters, C. M. and Bassler, B. L. 2005). These phenomena regulate bacterial response to local cell density and environmental stimuli.

Genes regulating hydrolytic enzymes and biofilm formation are controlled by QS in the phytopathogens *E. carotovora* and *P. stewartii* (Koutsoudis, M. D., et al. 2006; Whitehead, N. A., et al. 2002). We hypothesize that the QS locus we have identified are involved in regulating both virulence and rubrifacine production in walnut. Br-212 was HR minus on tobacco and

attenuated in virulence on walnut (Figure 1) indicating that genes regulated by this locus are important in these phenotypes.

Bacterial polymerases require the transient association of proteins called sigma factors for the initiation of transcription. Various sigma factors confer different specificities to the RNA polymerase allowing for the transcription of different genes. The open reading frame disrupted by transposon insertion was homologous at the nucleotide level to the factor  $\sigma_{54}$  or *rpoN* (Table 1).  $\sigma_{54}$  belongs to an exclusive class of sigma factors, distinct from broad class sigma factors also called the  $\sigma_{70}$  family (Buck, M., et al. 2000;Kazmierczak, M. J., et al. 2005). In some bacterial species,  $\sigma_{54}$  factors regulate nitrogen and carbon utilization (Cases, I., et al. 2003). In the bacterial phytopathogen, *Pseudomonas syringae* pv. *Maculicola* the *rpoN* homolog is required for elicitation of the HR in tobacco leaves and virulence related symptoms in *Arabidopsis thaliana* leaves (Hendrickson, E. L., et al. 2000;Hendrickson, E. L., et al. 2000). This report shows that disruption of the *rpoN* homolog of *B. rubrifaciens* also abolishes HR elicitation in tobacco leaves and attenuates necrotic symptoms on tissue cultured walnut plants.

We have identified two different genetic loci have been that affect development of the HR in tobacco and necrotic symptoms on tissue cultured walnut plants. Further characterization of these genetic loci will facilitate our understanding of the extended latency period of *B. rubrifaciens* and accelerate the development of novel approaches to control deep bark canker in any walnut cultivar.

## REFERENCES

- Buck, M., Gallegos, M.-T., Studholme, D. J., Guo, Y. & Gralla, J. D. (2000). The bacterial enhancer-dependent sigma54 (sigmaN) transcription factor. *Journal of Bacteriology*, 182, 4129-4136.
- Cases, I., Ussery, D. W. & de Lorenzo, V. (2003). The sigma 54 regulon (sigmulon) of *Pseudomonas putida*. *Environmental Microbiology*, 5, 1281-1293.
- Feistner & Budzikiewicz, H. (1985)The structure of rubrifacine.*C. J. Chem.*, 63, 495-499.
- Gera, C. & Srivastava, S. (2006). Quorum-sensing: The phenomenon of microbial communication. *Current Science (Bangalore)*, 90, 666-676.
- Hendrickson, E. L., Guevera, P. & Ausubel, F. M. (2000). The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of hrpL transcription. *J. Bacteriol.*, 182, 3508-3516.
- Hendrickson, E. L., Guevera, P., Penaloza-Vazquez, A., Shao, J., Bender, C. & Ausubel, F. M. (2000). Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is *rpoN* dependent. *Journal of Bacteriology*, 182, 3498-3507.
- Kazmierczak, M. J., Wiedmann, M. & Boor, K. J. (2005). Alternative sigma factors and their roles in bacterial virulence. *Microbiology and Molecular Biology Reviews*, 69, 527-533.
- Koutsoudis, M. D., Tsaltas, D., Minogue, T. D. & von Bodman, S. B. (2006). Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *PNAS*, 103, 5983-5988.
- McClean, A. E., Sudarshana, P. & Kluepfel, D. A. (2006) Development of PCR-based method for the detection of *Brenneria rubrifaciens*; the causal agent of deep bark canker of walnut in *Walnut Research Reports 2005* pp. 327-331

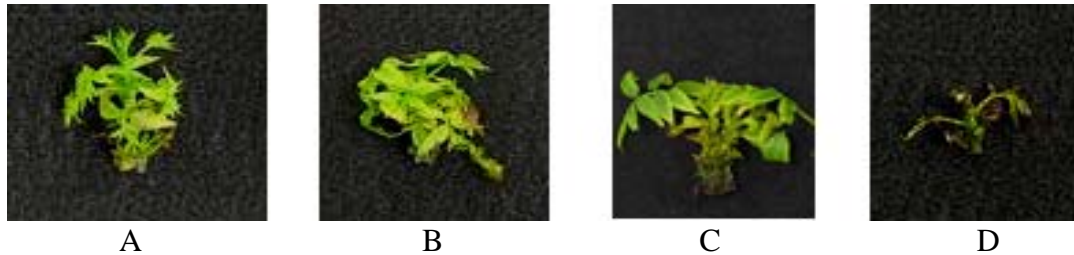
Waters, C. M. & Bassler, B. L. (2005). Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology*, 21, 319-346.

Whitehead, N. A., Byers, J. T., Commander, P., Corbett, M. J., Coulthurst, S. J., Everson, L., Harris, A. K. P., Pemberton, C. L., Simpson, N. J. L., Slater, H., Smith, D. S., Welch, M., Williamson, N. & Salmond, G. P. C. (2002). The regulation of virulence in phytopathogenic *Erwinia* species: Quorum sensing, antibiotics and ecological considerations. *Antonie van Leeuwenhoek*, 81, 223-231.

<i>B. rubrifaciens</i> Mutants	Locus Description	Database	E-value
Br-212, Br-512	echI (OHHL synthesis protein)	NCBI nucleotide collection (nr/nt)	$5 \times 10^{-77}$
Br-415	RNA polymerase $\sigma$ factor 54 <i>Erwinia carotovora</i> subsp. <i>atropetica</i> SCRI1043	NCBI Genomic Reference Sequences	0.0

**Table 1. Sequence analysis of *B. rubrifaciens* mutants attenuated in virulence.**

Plasmids generated from genomic DNA of *B. rubrifaciens* mutants were sequenced into the host DNA flanking the transposon. The DNA sequence was analyzed using vector NTI software and nucleotide (BLASTn) from NCBI.



**Figure 1. Bioassay on Walnut Plants.**

Tissue cultured walnut plants were inoculated with *B. rubrifaciens* mutants. Plants were infected using a sterile scalpel containing A) no bacteria B) mutant Br-415 (*rpoN*-like mutant) C) mutant Br-212 (*expI*-like mutant), and D) wildtype *B. rubrifaciens* 6D370. Photos were taken 1 month post inoculation.