

13.17 A transgenic approach to the control of citrus greening

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Genetic engineering of plants provides a powerful new technology for plant disease control that goes far beyond the cloning and transfer of natural resistance genes that are used by traditional plant breeders. For example, recent success in the control of virus diseases of plants by genetic engineering does not utilize traditional plant resistance genes against the viruses. Since each gene construct to be used in a food plant must undergo a significant set of regulatory hurdles (and expense), it is commercially impractical to develop multiple, highly specific gene constructs to control multiple plant diseases. In addition, **controlling disease but not killing the pathogen is not commercially acceptable because it would then allow transgenic plants to be hidden carriers of disease that would affect nontransgenic plants of the same species.** This would create additional regulatory and shipping problems, requiring segregation of transgenic from nontransgenic plants. Therefore IPG's strategy is to develop a single technology that can serve as a platform to control multiple plant diseases in a variety of plants, in this case, citrus greening, caused by a Gram negative bacterial pathogen, *Ca. Liberibacter asiaticus* (Las).

Background: bacteriophage are safe, effective, and lethal to bacteria. Bacteriophage have a long history of safe and effective use against Gram negative bacterial pathogens (Flaherty et al. 2000). In all cases using intact bacteriophage, the phage must first attach to the bacterial host, and that attachment is highly host specific, limiting the utility of the phage to specific bacterial host species, and sometimes specific bacterial host strains. In addition, for attachment to occur, the bacteria must be in the right growth phase, and the phage must be able to gain access to the bacteria, which are often buried deep within tissues of either animals or plants, or shielded by bacterial biofilms. Since Las is phloem intracellular and phloem-limited, it would be impossible to deliver phage to attack this target. However, if the proteins made by phage to kill bacteria or to compromise bacterial defenses are identified, a transgenic approach could be used to deliver these proteins to the target with great efficiency and consistency.

Isolation of phage with ability to affect non-host bacteria and genomic sequencing. Unsterilized pond water taken from an agricultural setting was used to isolate phage using *X. pelargonii*. Plaques were observed after 24 hrs. incubation; 24 of these were collected by scraping the plaques from the plates, titered and stored. These mixtures of phage were then purified from single plaques. Cell suspensions of overnight broth cultures of *X. citri*, *X. campestris* and *R. solanacearum* were added to 0.7% water agar and individually overlaid on phage-infected *X. pelargonii* plates. Plates were incubated an additional 48 hrs at 30° C and phage were evaluated for ability to kill or affect growth of Gram negative bacteria that they could not infect. Two such phage were selected, phage isolates 13 (Xp13) and 15 (Xp15)

The Xp15 genome was completely sequenced in order to identify the gene(s) expressing the diffusible killing factor (Ramadugu, Reddy and Gabriel, unpublished). Xp15 DNA was made according to standard protocols using *X. pelargonii* as the host. The Xp15 DNA was

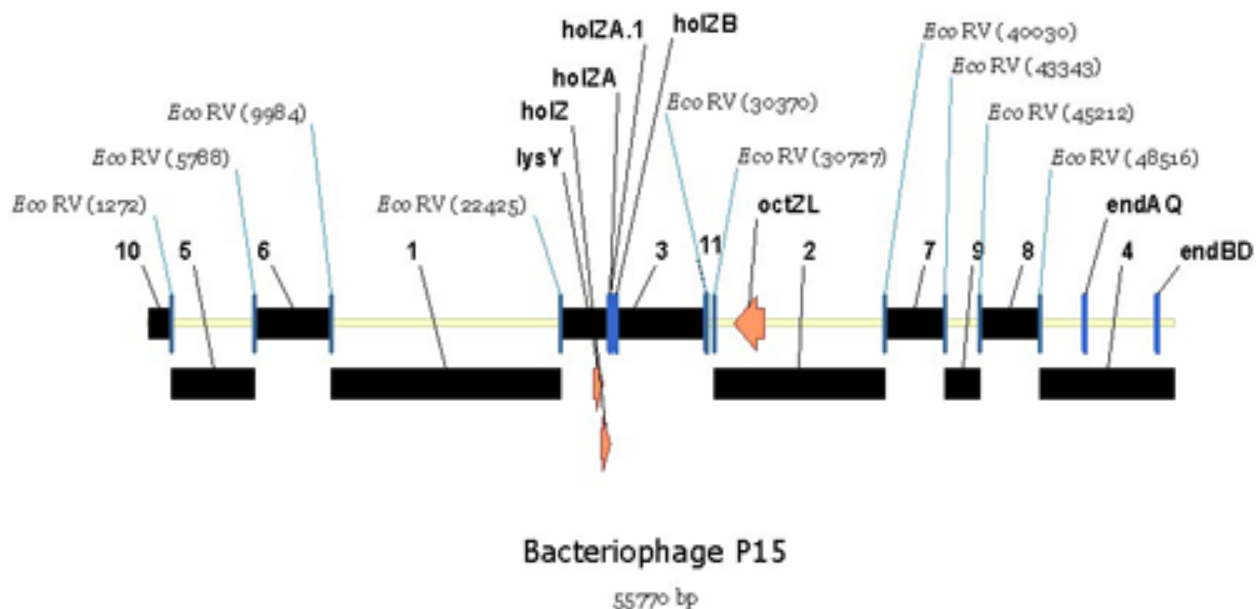


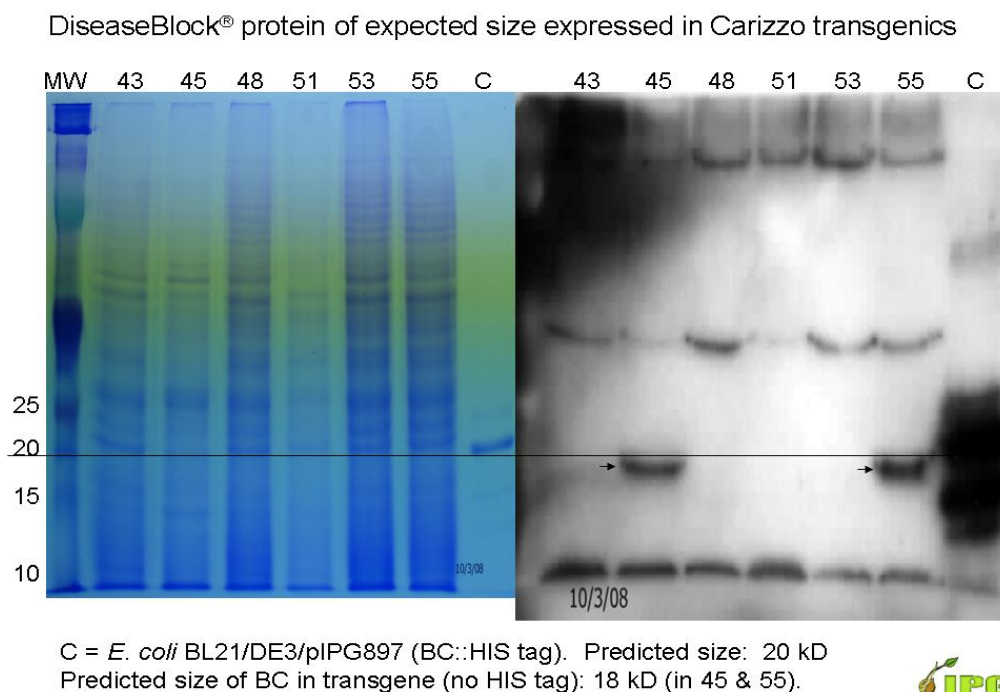
Figure 1 digested with *EcoRV*, yielding eleven fragments, ranging in size from 12.4 kb to 357 bp. The DNA fragments were mapped (refer Fig. 1). Most of the fragments were cloned; some were not cloned, despite repeated attempts. The cloned DNA fragments were used directly for sequencing, using vector-based primers initially, and primer walking thereafter until each fragment was completed. Fragments that were not cloned were sequenced using Xp15 genomic DNA. Fragment assembly was accomplished using Xp15 genomic DNA and primers extending outside each fragment in both directions. Xp15 has a double stranded DNA genome which is 55,770 bp in length.

ORF analysis of the sequenced phage was done using a combination of several programs including PromScan, Terminator (GCG), GeSTer (Unniraman et al. 2001, 2002), Glimmer, Genie, Codon preference (GCG), ORF finder (NCBI) and Blast (NCBI) analyses. Potential Shine-Delgarno sequences were identified manually by examining the sequence. Using default Glimmer settings, only 32 ORFs were identified. After identifying the promoters and terminators in the genome, manual analysis of all ORFs using Codon preference (GCG) allowed the identification of an additional 52 ORFs. The genome encodes 84 putative ORFs, including 3 holins (needed by the phage to break through the inner bacterial membrane) and an endolysin (needed by the phage to break through the cell wall). All four of these genes were cloned into a tightly regulated expression vector and expressed in *E. coli* BL21 DE3, and functioned as expected: when each holin was expressed in *E. coli*, growth ceased almost immediately upon induction; when the endolysin and holin were expressed together, cell lysis occurred.

Identification of a novel bacteriophage gene, “B” that caused “quasi-lysis”. Several other interesting candidate genes were cloned in the same vector and analyzed in the same way; one of them, candidate “B”, caused a slow “quasi-lysis”. When combined with an endolysin, there was no lysis, indicating its primary effect was not on the inner membrane. Microscopic examination of cells expressing protein B showed near cessation of cell division, and many of the cells

appeared elongated, as if many cells failed to divide. Polyclonal antisera was raised against the Xp15 B protein overexpressed in *E. coli*.

Transgenic tobacco and citrus expressing Xp15 “B”. The gene encoding Xp15 B was recloned into a binary plant transformation vector and used to transform both tobacco and citrus Carrizo citrange. Forty four confirmed transgenic tobacco lines were generated and rooted from 235 leaf explants (19%). Fifty five confirmed transgenic Carrizo lines were generated and rooted from 1678 shoot explants (3%). Southern, Northern and Western blot analyses were used to confirm gene expression. Thirty of these confirmed lines were evaluated by Western blot analysis and 10 of these plant lines were confirmed to strongly express the Xp15 B protein:



Las challenge inoculations on tobacco and citrus. Sixteen of the confirmed transgenic tobacco lines and 5 of the confirmed transgenic Carizzo lines were moved into the UF/ICBR Plant Containment Facility for challenge inoculations. This Facility was certified for use with the Las Select Agent. Using dodder transmission from an HLB infected citrus tree provided by FDACS-DPI, HLB was transmitted to both periwinkle (*Vinca*) and to sweet orange. Transgenic tobacco carrying Xp15 B was challenge inoculated with strongly Las + dodder using HLB "strain", UF506 in four experiments beginning in July, 2007. Results using tobacco as a proxy host for Las were as follows:

| | Exp. | Dodder | | |
|---------|------|-----------|------------|------------|
| | | On | Attached | Off |
| Tobacco | 1. | 7/25/2007 | 10/17/2007 | 11/29/2007 |
| Tobacco | 2 | 1/18/2008 | 1/28/2008 | 3/31/2008 |
| Tobacco | 3 | 3/18/2008 | 4/29/2008 | 6/19/2008 |
| Tobacco | 4 | 4/18/2008 | 4/29/2008 | 6/19/2008 |

| | | | |
|---------|-----------|-----------|-------------|
| Tobacco | | | |
| Exp. | 4 | | |
| Tobacco | 6/21/2008 | 7/19/2008 | 8/15/2008 |
| NT | | 8/26 | (30%) Las + |
| Xp15 B | | 0/16 | (0%) Las + |

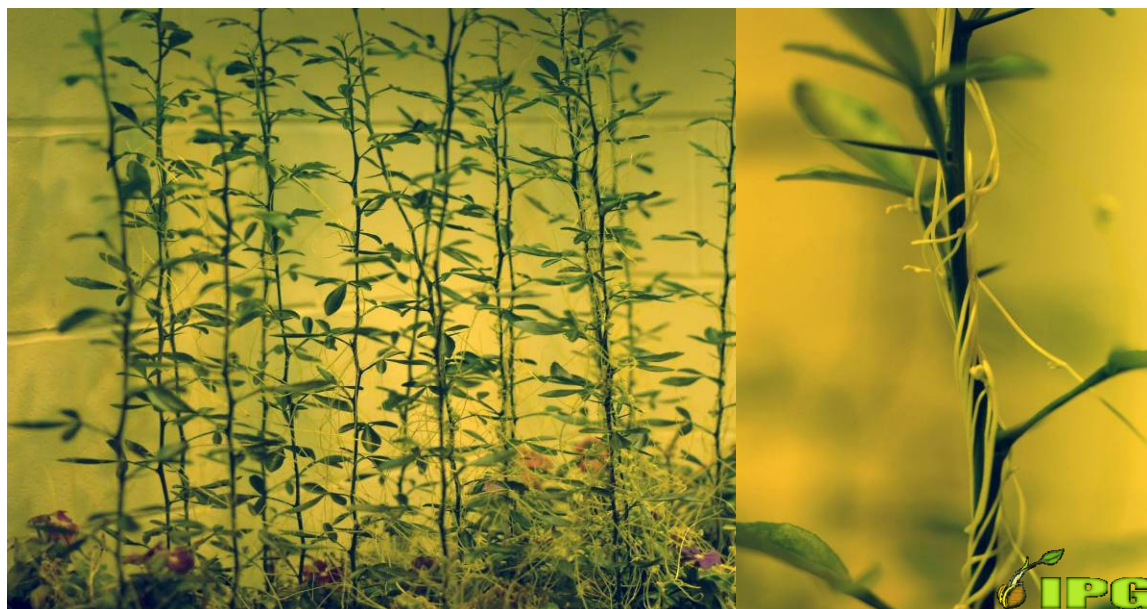
Summary All Tobacco

Expect: 4.9/16 Xp15 B plants to be Las +, if no resistance.

Since 8/26 experimental tobacco plants (30%) were Las positive, if no resistance were found in Xp15 B plants, one would expect 4.9/16 of these plants to be Las positive, but none of the Xp15 B plants were positive. This result indicates that transgenic tobacco carrying Xp15 B are resistant to Las. The experiment was conducted by using dodder to transmit Las from confirmed, infected sweet orange as a source to periwinkle (*Vinca*). After the periwinkles became symptomatic and were confirmed by PCR to be Las +, the dodder was detached from citrus but not from periwinkle, and the infected periwinkles and infected dodder were placed in very close proximity to the tobacco. The dodder was trained to grow on tobacco. After the dodder was confirmed attached to the tobacco plants (usually after 6 weeks), the dodder was allowed to remain on the tobacco for at least 4 weeks, as indicated in the table.



Similar experiments were conducted with transgenic citrus. Transgenic Carizzo citrange carrying Xp15 B was challenge inoculated with strongly Las + dodder using HLB "strain", UF506 in four experiments beginning in April, 2008. Results using these transgenic citrus plants were as follows:



| | Dodder | | |
|--------------|-----------|-----------|-----------|
| | On | Attached | Off |
| Exp 1 Citrus | | | |
| Carrizo | 4/11/2008 | 4/18/2008 | 5/19/2008 |
| Exp. | 2 | | |
| Citrus | | | |
| Carrizo | 5/20/2008 | 5/27/2008 | 6/24/2008 |
| Exp. | 3 | | |
| Citrus | | | |
| Carrizo | 4/18/2008 | 4/29/2008 | 6/19/2008 |
| Exp. | 4 | | |
| Citrus | | | |
| Carrizo | 6/21/2008 | 8/1/2008 | 9/23/2008 |

Summary All Carizo

NT 3/26 (11.5%) Las +
Xp15 B 0/14 (0%) Las +

Expect: 1.6/14 Xp15 B plants to be Las +, if no resistance.

Since 3/26 experimental Carrizo plants (11.5%) were Las positive, if no resistance were found in Xp15 B Carrizo, one would expect 1.6/16 of these plants to be Las positive, but none of the Xp15 B Carrizo plants were positive. This result indicates that transgenic citrus carrying Xp15 B is resistant to Las.

Conclusions. Citrus greening is a devastating plant disease that has spread throughout Florida and is now in all citrus growing counties. The lag time between the first introduction of the citrus psyllid insect vector (1999 in Florida) to the confirmation of citrus greening disease (2005 in Florida) is about six years. The psyllid is now in Texas and California. There is no practical resistance breeding in citrus, and eradication of the psyllid or greening is impossible. Chemical control of the insect vector may require up to 25 sprays per year, and this only suppresses the disease. Citrus greening is caused by Las, an intracellular, Gram negative bacterium that resides within the living phloem cell. A genetic engineering approach to introduce a gene that allows the plant to defend its own cells from within is most likely to succeed. We have evidence that a bacterial phage gene, when expressed inside both tobacco and

citrus plant cells, produces a stable protein that is not harmful to citrus and appears to give resistance to Las, and therefore the disease it causes, citrus greening.

We are in the process of obtaining regulatory agency approvals and moving these genes into mature Hamlin and Valencia tissue.

Citations

Flaherty JE, Harbaugh BK, Jones JB, Somodi GC, Jackson LE. 2001. H-mutant bacteriophages as a potential biocontrol of bacterial blight of geranium. *HortScience* 36 : 98-100.

Unniraman S, Prakash R, Nagaraja V. 2001. Alternate paradigm for intrinsic transcription termination in eubacteria. *Journal of Biological Chemistry* 276:41850-41855.

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