AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2015 – 2017

TITLE: Improved biological control of crown gall disease on nursery plants

RESEARCH LEADER: Walt Ream

COOPERATORS: none

SUMMARY:

Agrobacterium tumefaciens, a ubiquitous soil bacterium, causes crown gall tumors in fruit and nut trees, grapes, and other nursery crops, resulting in millions of dollars of damage annually. *A. tumefaciens* transfers tumor-causing genes into plant cells, which then grow into galls. After a few hours of infection, the disease progresses even if the tumor-inducing bacteria are killed. Prevention is the only effective way to control crown gall.

Adequate means do not exist to prevent crown gall. Inoculation of plants with *Agrobacterium radiobacter* K84 controls a limited number of *A. tumefaciens* strains, but crown gall remains a serious problem. We created crown-gall-resistant apple trees by introducing a gene that silences the tumor-causing genes (oncogenes) of *A. tumefaciens*. This technology is highly effective, but it is not economically practical because each plant species must be genetically modified with our gall-resistance (oncogene-silencing) gene. The resulting plants are genetically modified (GMOs) and require federal approval prior to commercial use. Testing required for approval costs millions of dollars.

Our first-generation gall-resistance gene protected grapevines against some (but not all) strains of *Agrobacterium vitis* and *A. tumefaciens*; oncogene sequence variability allowed some strains to escape gene silencing. Advances in gene silencing technology defined precisely the requirements for gene silencing. Colleagues developed vectors for efficient gene silencing in plants, which we used to build a new oncogene silencing construction predicted to silence oncogenes from most *Agrobacterium* strains. We created a novel strain of *Agrobacterium* that transfers our new gall-resistance gene into plant cells. We tested the ability of this biocontrol strain to prevent crown gall disease.

OBJECTIVES:

1. Insert highly conserved 21-base-pair oncogene sequences into a gene silencing vector.

2. Introduce this gene silencing vector containing the gall-resistance gene into a non-pathogenic *Agrobacterium* strain that can efficiently transfer the gall-resistance gene into plants.

3. Test the efficacy of our novel biological control strain in laboratory trials.

PROCEDURES:

Aim 1. We used standard molecular genetic methods to fuse a highly conserved oncogene sequence into the gene silencing vector. We confirmed the sequence of the gene construction.

Aim 2. We moved the gene silencing vector into a non-pathogenic "disarmed" strain of *Agrobacterium*, which lacks oncogenes but can transfer DNA to plant cells.

Aim 3. We compared crown gall incidence on mock-inoculated carrot root discs and on carrots inoculated with: a) biocontrol strain, b) "disarmed" strain (without the gall-resistance gene), c) biocontrol strain and pathogenic *A. tumefaciens* (1:1, 3:1, and 6:1 ratios of biocontrol strain to pathogen), d) "disarmed" strain and pathogen (1:1, 3:1, and 6:1 ratios), and e) pathogenic *A. tumefaciens.* Tumor foci were assessed after one month.

SIGNIFICANT ACCOMPLISHMENTS:

To identify highly conserved target sequences for silencing, we aligned *iaaM* oncogene sequences from 54 *Agrobacterium* strains. Sequences included *iaaM* genes from 43 *A. tumefaciens*, 4 *A. vitis*, 3 *A. rhizogenes*, and 4 strains of a new *Agrobacterium* species whose genomes were sequenced by our laboratory. Eleven of these sequences came from public databases, whereas 43 genes were sequenced at OSU in the Chang and Ream laboratories. Our analysis identified nine potential target sequences expected to silence *iaaM* genes from 49 strains, including two of the *A. vitis*. One of these sequences also targets *iaaM* from a third *A. vitis* strain, bringing the total to 50 *Agrobacterium* strains that may be controlled by one gall-resistance gene.

We inserted the *iaaM* target DNA into the gene silencing vector and performed DNA sequence analysis, confirming that the sequence of the oncogene-silencing transgene is correct. To construct a biocontrol strain, we transformed the vector containing the *iaaM* target sequence into a non-pathogenic *Agrobacterium* strain designed to deliver the oncogene-silencing transgene to plants.

We tested the ability of the biocontrol strain to prevent crown gall tumors on carrot root discs inoculated simultaneously with pathogenic *A. tumefaciens* and the biocontrol strain (Figure 1). Tests were performed on two cultivars of fresh (leaves on), organically grown carrots. Prior to inoculation, roots were washed with detergent, rinsed with ethanol, and surface sterilized by soaking in 20% bleach + detergent for 20 minutes, followed by a rinse with sterile distilled water. Roots were sliced into discs under aseptic conditions and placed in Petri dishes containing agar; basal surfaces were inoculated with log-phase bacterial cultures. All comparisons were made between serial slices taken from the same carrot. We performed two tests (on separate days) with replicates on five carrots per experiment (ten carrots total). The biocontrol strain did not prevent tumor formation on carrot when inoculated simultaneously with the pathogen. We plan to test whether inoculation with the biocontrol strain prior to infection by pathogenic *A. tumefaciens* can prevent tumor formation. In nurseries, growers treat plants with biocontrol agents prior to planting and potential exposure to *Agrobacterium*, so this strategy is feasible.

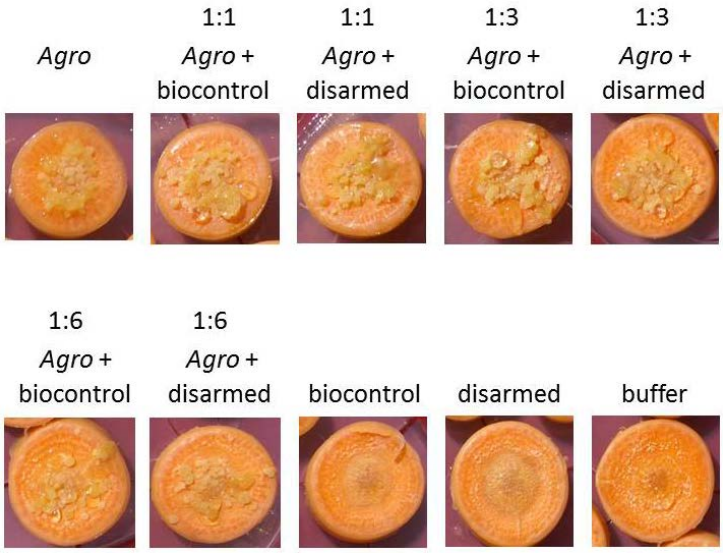

Figure 1. Virulence assays on carrot root discs. Carrots discs inoculated with: pathogenic *A. tumefaciens* (*Agro*), non-pathogenic *Agrobacterium* with oncogene silencing gene (biocontrol), non-pathogenic *Agrobacterium* (disarmed), or buffer. Mixtures of pathogenic *Agrobacterium* and non-pathogenic biocontrol or disarmed strains were applied in 1:1, 1:3, and 1:6 ratios of pathogen:non-pathogen.

Positive controls (discs inoculated only with pathogenic *A. tumefaciens*) on three carrots showed vigorous tumor growth one month after inoculation, as expected (Figure 1). However, discs from seven carrots did not respond to infection by the pathogen (not shown), which is highly unusual. At least one carrot from each cultivar responded typically, ruling out the possibility that one of the varieties is naturally resistant to crown gall. In each experiment, all carrots were inoculated using the same bacterial cultures, ruling out differences in the inoculum. Bacteria that naturally inhabit the interior of the gall-resistant carrots may have prevented infection by pathogenic *A. tumefaciens.*

To investigate this possibility, we cultured a novel bacterial species from the basal surface of a tumor-free disc previously inoculated only with pathogenic *A. tumefaciens*. Sequence analysis of a portion of this organism's small subunit ribosomal RNA (SSU rRNA) gene revealed a new species of *Pseudomonas* whose SSU rRNA gene shares ~79% DNA sequence identity with its

closest known relatives. We plan to test whether this organism can prevent infection by pathogenic *A. tumefaciens.* We also plan to sequence the genome of this novel species.

To evaluate the effectiveness of our oncogene-silencing transgene in transgenic plants, we introduced the transgene into *Arabidopsis thaliana*. First-generation (T0) seeds are currently under selection (for resistance to hygromycin) to identify transgenic lines. We will characterize transgene expression, structure, and copy number. Lines that express the transgene will be inoculated with pathogenic *A. tumefaciens* to assess the effectiveness of the oncogene-silencing transgene.

BENEFITS & IMPACT:

Although simultaneous inoculation with biocontrol and pathogenic strains did not prevent crown gall disease, colonization of the host with the biocontrol strain prior to infection by the pathogen may provide protection. Similarly, incorporation of the oncogene-silencing construction in transgenic plants may protect the transgenic plants from crown gall disease. We plan to test both possibilities.

Our unexpected discovery of a novel *Pseudomonas* species living inside carrot roots that exhibited resistance to crown gall disease may offer another means for biological control of crown gall. We plan to test whether this bacterium is responsible for the disease resistance we observed. If so, this bacterium offers a biocontrol strategy free of regulatory hurdles associated with deliberate release of transgenic organisms into the environment.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: none

FUTURE FUNDING POSSIBILITIES: USDA