Final report, Virginia Wine Board FY 2017

"Validation of *Rhizobium vitis* ARK-1, a biological agent against crown gall of grape"

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Objectives: We propose to continue an international research collaboration with Dr. Kawaguchi's lab in order to develop a sound management program against crown gall. The major objectives for this year are:

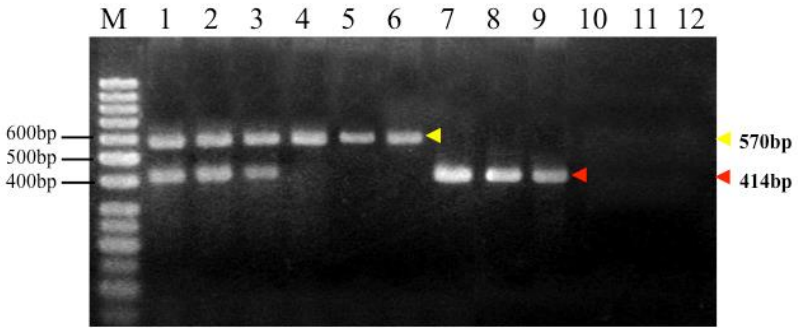
1) Continued isolation and identification of *R. vitis* populations from VA vineyards;
2) Validation studies on ARK-1 strain against VA strains in the green house and field conditions;
3) Screening for VA native non-pathogenic *R. vitis* strains for potential use as biological agents; and
4) Understand VA native *R. vitis* population structure on a genetic and geographical level.

Summary of procedures

During 2015 and 2016, we visited ten VA vineyards and collected over 60 galls form grapevines with crown gall symptoms. Isolation of *R. vitis* was conducted and over 700 individual bacterial colonies have been isolated. In 2016, a graduate research associate, Mr. Alex Wong, optimized a multiplex PCR procedure developed by Kawaguchi (2005) to identify these bacterial colonies to determine whether they are 1) *R. vitis* or not, and if it is, 2) whether *R. vitis* contains a piece of DNA that cause gall or not.

The multiplex polymerase chain reaction (PCR) is performed using a mixture of two primer sets; a forward primer Ab3-F3 (5'-ATG ACG GTA GTC GGA GAA GAA GCC-3') paired with a reverse primer Ab3- R4 (5'-CTG TCT CTG TGT CCC CGA AAG G-3') and a forward primer VCF3 (5'-GGC GGG CGY GCY GAA AGR AAR ACY T -3') paired with a reverse primer VCR3 (5'-AAG AAC GYG GNA TGT TGC ATC TYA C-3'); to identify tumorigenic and nonpathogenic strains of *Rhizobium vitis*. DNA fragments (414 bp) from a partial sequence of the *virC1* and *virC2* genes encoded in the *virC* operon is expected to be amplified from the cell lysate of tumorigenic or rhizogenic *Rhizobium* strains by PCR with VCF3 and VCR3 primers, and 570-bp fragments from 16S rDNA is expected to be amplified from *R. vitis* strains by PCR with Ab3-F3 and Ab3-R4 primers.

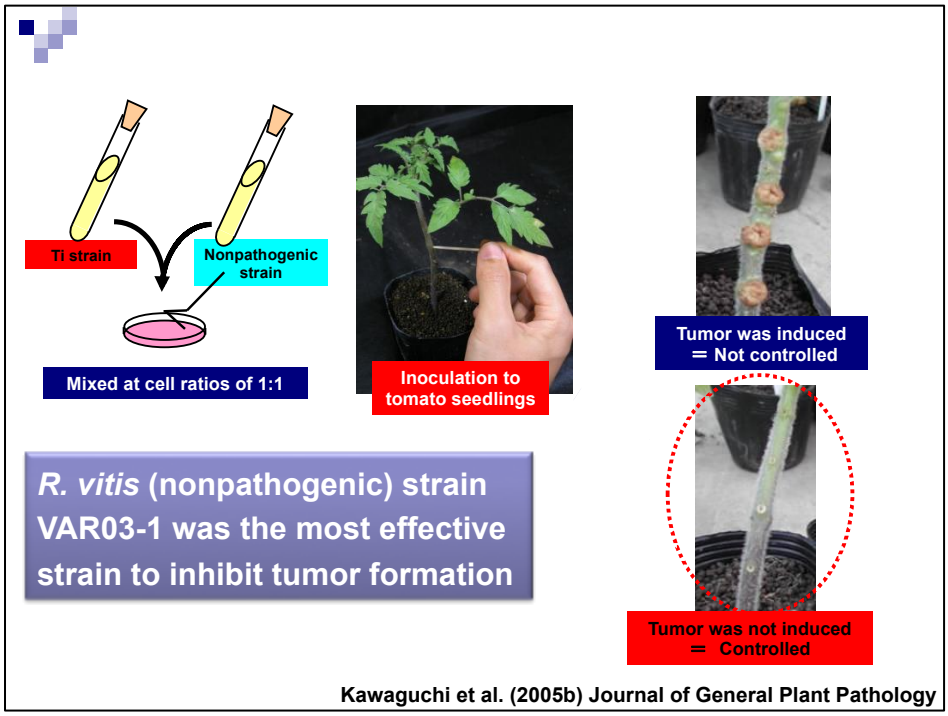
The multiplex PCR amplification of the target sequences is performed in a total volume of 25µl of the following reaction mixture: 1µl of template, 1µl of VCF3/VCR3 primer set (20pmol/µl), 1µl of Ab3-F3/Ab3-R4 primer set (5pmol/µl), 12.5µl of Qiagen Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), and 9.5µl of sterile distilled dnase free water. The multiplex PCR is performed in a PCR



thermal cycler after initial denaturation at 95°C for 14.5min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 90s, extension at 72°C for 90s, and an additional extension at 72°C for 10min.

The figure above shows examples of amplification products obtained by the multiplex PCR separated by agarose (3%) gel electrophoresis. Lanes: *M*, 50-bp DNA ladder. *Lane 1-3:* tumorigenic *Rhizobium vitis*. *Lane 4-6:* nonpathogenic *R. vitis*, *Lane 7-9:* tumorigenic *R. radiobacter* or tumorigenic *R. rhizogenes*. *Lane 10-11:* nonpathogenic *R. radiobacter* or nonpathogenic *R. rhizogenes*. *Lane 12*, water negative control. The expected positions of the *570-bp* and *414-bp* fragments are shown.

Once isolates were identified as tumorigenic *R. vitis* (e.g., samples 1-3 in the figure above), these isolates are validated with an inoculation to tomato plant to validate the results from the multiplex PCR procedure. Young beefsteak tomato plants (1-month-old) will be used for gall formation test. The bacterial colony grown on Roy and Sasser (RS) media will be grown in Yeast extract mannitol (YEM) broth, and inoculated into tomato by stabbing the stem and introducing the live cells to the wound. Each of tomato plant will receive four inoculations. Gall formation will be assessed with calipers measuring in millimeters at 4 and 6 weeks after inoculation for galls. The experiment will be repeated three times in greenhouse conditions.



Progresses made

During the summer of 2016, the multiplex PCR was conducted on all the isolates we obtained during 2015-16, and more than 80 *R. vitis* isolates were confirmed. Among these, we selected five isolates that were isolated from different part of the state to conduct more experiments.

We received a pre-commercial ARK-1 product in April 2016. Currently, we are in process of examining these five isolates by inoculating tomato with ARK-1 to see the one-on-one efficacy of ARK-1 against these VA-native *R. vitis* (Figure above). The cell suspensions of pathogenic and nonpathogenic strains are prepared from 48 hour old YEM broth tubes and adjusted to a certain optical density: OD600 = 0.1 (corresponding to about 10^8 cells/ml, then mixed in various combinations at cell ratios of 1:1 just before inoculation. A 5-µl drop of a strain alone or of a mixture will be dropped onto a needle-prick wound on the stem of a tomato or grapevine seedling. There are five inoculations per plant. The assessment of gall formation measured in millimeters will be conducted 28 and 42 days after inoculation.

So far our early results showed about 30% reduction of gall development. The number was far less than what Kawaguchi reported. Upon examining our experimental procedure, we found that the formulation we obtained from the chemical company was different because it contains adjuvants that alter our optical density measurement. Thus, the cell ration was no 1:1. This results probably indicate two important aspects of this method. 1) Dose matters: Lower dose most likely result in lower efficacy of the ARK-1, and 2) Even with a low dose, there is some efficacy, indicating the potential strong efficacy of the ARK-1. As of January 2017, we are repeating experiment with media-grown ARK-1 to make sure we can achieve 1:1 ratio.

Also, what we found out was a potentially limited movement by ARK-1. When we co-inoculate at one point of a tomato stem, and then inoculate only with a wild-type only 5 mm away from the point of co-inoculation, the point with wild-type only inoculation most likely develop a gall. It may also depend on the concentration of the ARK-1, and we would like to repeat the experiment with different concentrations of the ARK-1.

Future direction

Once we confirm the efficacy, we will select five 'lab' isolates that are very active on gall formation and preferably isolated from different geographic area. Then mixtures of isolates will be tested in the second round. Mixed inoculation will be conducted with single, double, triple, quadruple, and quintuple combinations (= 21 total combinations), *R. vitis* ARK-1 alone as a control, and water control (= 23 total). Plus, we will have VA isolate w/ *R. vitis* ARK-1 and VA isolate by itself inoculation. There will be two plants per isolate, and three experimental repetitions. Thus, a total of 252 tomato plants (21 combinations x 2 plants x 2 inoculations x 3 reps) will be used.

After we have established the efficacy of *R. vitis* ARK-1 with tomato, we will conduct a greenhouse experiment with grapevine seedlings (grown from seeds) of cv. Chardonnay and Cab Franc in the same manner to validate the results from tomato study. We expect that grapevine and tomato results will be nearly identical, but it will take more time (4 weeks vs. 3 months) to obtain the results with grapevines.

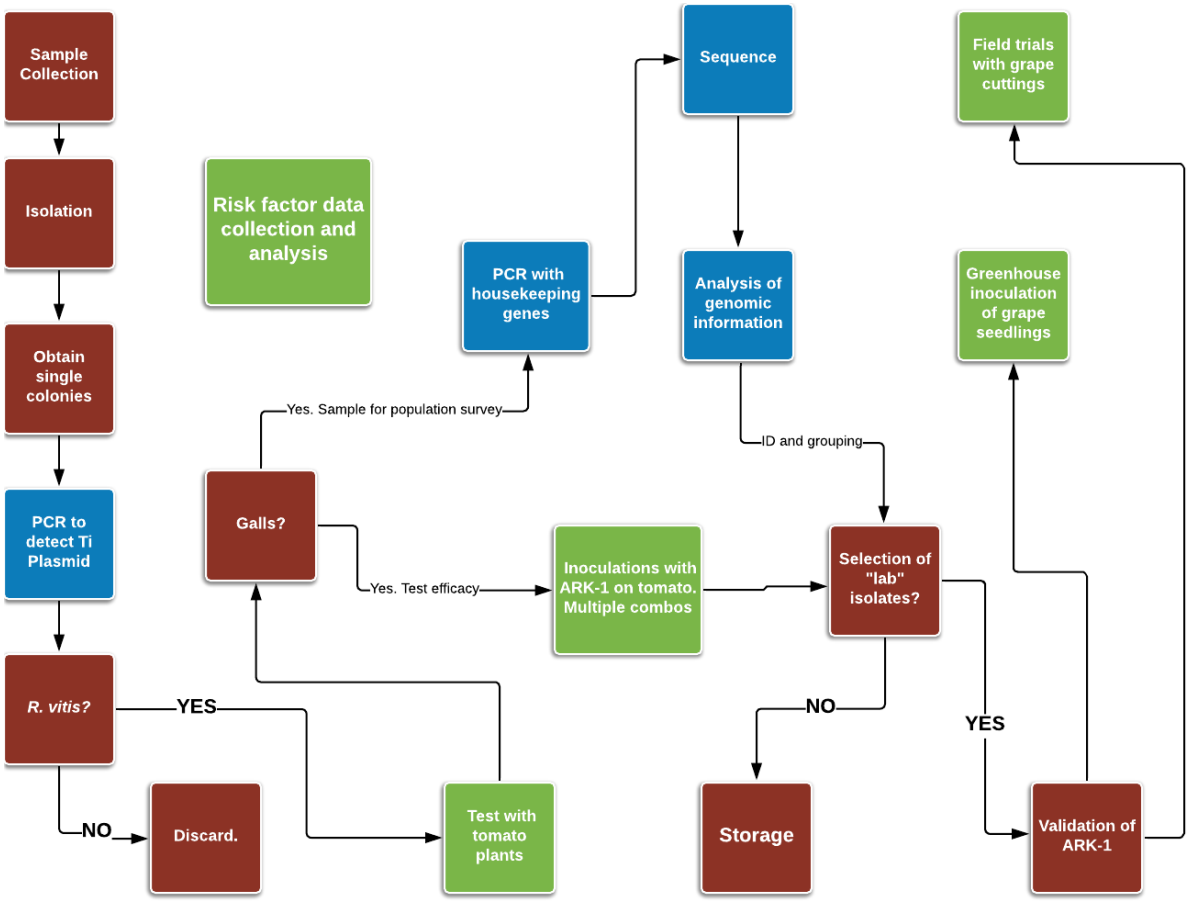
In addition, we have been performing a field trial where two of the five lab isolates, *R. vitis* ARK-1, and water control were tested with positive (w/ *R. vitis* ARK-1) and negative (VA isolate by itself). Four ARK-1 inoculation methods total (3 pre- and 1 post-inoculation): stabbing green tissue, drilling woody tissue, root dipping; and an after-inoculation bark spray application have been examined. Time frames between each inoculation will be one week. There are three plants per experiment, three experimental repetitions, and two cultivars (Chardonnay and Cabernet Franc) to be tested. Thus, a total of 160 plants (2 isolates x 8 plants x 5 inoculation methods x 2 cultivars) have been planted. Vine cuttings was obtained from our vineyards. Vines will be rooted in greenhouse, and then planted in new raised beds in AHS-AREC in a pot-in-pot system. Measures have been taken to prevent spread of both the pathogen and biocontrol agent to other grape cuttings. Experiment started in mid-May, and observation of gall formation will be recorded at the end of the season (Oct 2017).

Screening for biological agent and understanding *R. vitis* population in VA: The isolates that are identified as *R. vitis* without the tumor-causing plasmid will be subjected for further analysis because these are potential antagonistic strains that can be used in similar manner as in *R. vitis* ARK-1. The inoculation assay with tomato will be conducted to screen these isolates. If gall formation is not observed with co-inoculation with VA-isolates, DNA will be sequenced to examine the similarity to *R. vitis* ARK-1 and other known antagonistic strains (Figure below). Around one dozen isolates we identified so far lack the plasmid and will be candidates for the screening.

Another related objective with this screening process is understanding of VA-native *R. vitis* population. Based on our preliminary results, it seems that there is large range of variation on Ti-plasmid (the DNA that causes gall formation). Which could be a key to understand how our population(s) cause the disease, and hence, determine when/how to break the disease process. For example, *R. vitis* ARK-1 or other antagonistic strain may interact a certain function of gene that maybe unique to one population, but

not the others. This type of work requires accumulation of data, thus, we are not aiming to resolve in one year, but will be a good idea to start collecting data while we are working with many different isolates.

Geographic distribution of crown gall in Virginia vineyard will also be examined and correlated to a number of factors. This analysis of risk in respect to geography was of high interest to growers in the region. Relative elevation, slope, distance from a body of water etc. will be looked at to identify risk factors that may contribute to the incidence of crown gall. This study will be conducted in the fall of 2017 in collaboration with Alex Blackburn, a regional soil specialist, who will help with most of the geographical data.



Progresses made

Along with continuing to isolate and identify Virginia native *R. vitis* from galls, we have conducted our first round of tomato co-inoculations with ARK-1 and are currently analyzing the data from those experiments. The second round of tomatoes have been planted and should be ready to be inoculated by the end of August. Grape cuttings have also been inoculated and gall ratings for those plants should be ready by mid-October.

Other achievements

Teaching component has been the strength of the project. A master's level student, Mr. Alex Wong joined my lab in Summer 2016. He has been making an excellent progress with his academic work and only needs to complete his departmental seminar course (Fall 2017) to finish his academic requirements (GPA 3.78). Alex has also fulfilled his teaching assistantship requirement on Virginia Tech campus.

Personnel

- Mizuho Nita, PhD (AHS AREC, PPWS, Virginia Tech): PI to oversee the whole operation, design the experiments, analyze data, supervise a master's level student.
- Akira Kawaguchi, PhD (Okayama Prefectural Government, Okayama, Japan): Collaborator to provide technical guidance.
- Mr. Alex Wong (AHS AREC, Virginia Tech): MS level graduate research associate. He has been working on lab, greenhouse, and field evaluations, and screening of potential biocontrol agent.
- Ms. Akiko Mangan (Research Associate, AHS AREC, Virginia Tech): Lab technician to assist PCR assays

Selected references

Kawaguchi A, Sawada H, Inoue K, Nasu H. (2005) Multiplex PCR for the identification of Agrobacterium biovar 3 strains. *Journal of General Plant Pathology* 71:54–59.

Kawaguchi A, and Inoue K. (2012) New antagonistic strains of non-pathogenic *Agrobacterium vitis* to control grapevine crown gall. *Journal of Phytopathology* 160:509–518.