Final Report

Development of a Magnetic Capture Hybridization Real-Time PCR Assay for Detection of *Agrobacterium* in Rose

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July 8, 2016

Introduction

Crown gall of rose is caused by the bacterium *Agrobacterium tumefaciens*. Disease results from natural gene transfer initiated by the bacterium and culminating in the insertion of fragments of plasmid-borne bacterial genes, including those for pathogenicity, which are integrated into the plant genome (12). Host cells undergo a series of metabolic changes and are induced to overproduce plant hormones, resulting in rampant unorganized cell proliferation and gall production (12). This often forms at wound sites, such as at the base of cuttings. However, it can be difficult to distinguish crown gall from normal callus production, especially in particularly vigorous rose cultivars. In field-grown roses, the bacteria are presumed to enter the roots from infested soil, after which the bacteria become systemic (11), moving from the roots to other parts of the plant. Secondary spread of the bacteria occurs when green cuttings are taken from symptomless plants which are nonetheless infected (14, 15). Galls often do not form in such cuttings until after they have been distributed in the nursery trade, which is why crown gall is considered one of the most damaging diseases (15), and poses a threat of serious economic liability for production nurseries. There is no cure, and currently diseased plants must be destroyed to prevent further spread. Losses due to *A. tumefaciens* have amounted to many millions of dollars annually in the United States (8). A consistently reliable method for the early confirmation of crown gall is critical for rapid mitigation of disease.

PCR is a molecular technique that uses small designer DNA probes called primers to amplify specific target sequences of DNA. Because primers can be synthesized relatively quickly and inexpensively, PCR can be easily adapted for the detection of a known DNA sequence in virtually any organism. For this reason, this technique is widely used in the diagnosis of both plant and animal diseases. Currently, there are multiple PCR primers available for detection of pathogenic *A. tumefaciens*, generally targeted to virulence genes located on the plasmid which confers pathogenicity.

Real-time PCR eliminates the need for gel electrophoresis used in traditional PCR since the real-time assay results are indicated by fluorescence of amplified product in the reaction mixture. The fluorescence is detected by a machine as it develops (the real-time aspect of the assay).

Both real-time and traditional PCR work well for many crops and ornamentals, but woody plants present a unique challenge. Woody tissues contain the structurally complex biopolymer lignin, which is associated with many compounds, such as polyphenols and polysaccharides, that are inhibitory to the molecular interactions required for successful PCR (7, 10). A common solution to this problem in research laboratories is the use of phenol and chloroform to separate DNA from co-extracted inhibitory compounds (17); however, the extremely hazardous nature of these chemicals and the requirement for costly equipment and laborious safety procedures when utilizing them makes this an impractical approach to high-throughput screening of large numbers of samples. Some techniques include the addition of “buffer enhancers” such as DMSO, Tween, PEG, or bovine-derived protein extracts to the

PCR reaction mixture (3, 9, 13). Some extraction methods call for the addition of CTAB to the extraction buffer at the step in which cells are ruptured to extract DNA (1, 17). PCR protocols may employ one or more of these techniques during DNA extraction, cleanup, or amplification to mitigate inhibition; however, we have found inhibitory compounds in rose to persist despite the use of both CTAB and buffer enhancers. The difficulty of detecting *A. tumefaciens* in infected rose stems is well known by those who work with rose and has been previously documented (2).

Magnetic capture hybridization (MCH) is a method for the isolation and purification of nucleic acids from substrates such as soil, food, and other complex samples, including plant tissues (4, 5, 7, 16). MCH uses short fragments of DNA attached to magnetic beads that, like the primers used in PCR, hybridize to target DNA sequences (10, 16). These hybrids can then be magnetically isolated and purified, and the captured DNA used for PCR applications. This method has previously been successfully employed to purify DNA from lignin-associated inhibitors present in woody tissues (7, 10). The development of a real-time PCR assay in combination with the MCH would result in an overall reduction in assay time and would simplify the entire procedure since visualization of amplification is not gel-dependent. These improvements in purification and detection of pathogenic *A. tumefaciens* DNA in rose tissue would be a dramatic enhancement of the sensitivity and reliability of the traditional PCR assay. Our objective for this project was to adapt the magnetic capture hybridization technique for the purification of DNA from symptomatic rose tissue, and to develop a real-time PCR assay for *Agrobacterium* that could be used to detect the pathogen in the MCH-purified DNA. In addition to screening symptomatic gall tissue, we will use this MCH-assisted real-time PCR assay to screen asymptomatic tissues from infected plants. We propose to test both root tissue and twig tissue from areas of the plant distal from the site of inoculation, to determine the efficacy and sensitivity of the assay in detecting systemic infections.

Materials and Methods

Bacterial strains, plant lines, and growth conditions

For this study, we used the highly susceptible rose cultivar “Head Over Heels” grown in controlled greenhouse conditions. For the inoculation of these roses, we used four pathogenic isolates of *Agrobacterium* previously isolated from disease samples received by the OSU Plant Clinic. Furthermore, DNA was isolated from 44 different strains of *Agrobacterium* for use in establishing the efficacy of our oligonucleotide probe in detecting pathogens from a wide variety of hosts, strains, and even different species of *Agrobacterium.* Some non-pathogenic strains of *Agrobacterium* were included as controls. All *Agrobacterium* strains were grown on MGY agar at 23°C. See Table 1 for a list of strains used in this study; where applicable, host and location of origin are included

Table 1. *Agrobacterium* strains used in this study.

Strain	Host	Location	Comment	Result
A. tumefaciens M2/73	Birch			Positive
A. tumefaciens K27	Poplar		Pathogenic	Positive
A. tumefaciens N2/73	Cranberry			Positive
A. radiobacter H4/72	Azalea		Non-pathogenic	Negative
A. radiobacter S5/72	Incense Cedar		Non-pathogenic	Negative
A. radiobacter U3	Willow		Pathogenic	Positive
A. radiobacter S9/73	Lippia		Pathogenic	Positive
A. tumefaciens C1/73	Sorbus		Non-pathogenic	Negative
Agrobacterium spp. 16/2	Grape	Hungary		Positive
Agrobacterium spp. CG59	Grape			Positive
A. radiobacter K9	Soil		Pathogenic	Positive
Agrobacterium spp. CG58	Grape			Positive
A. tumefaciens G5/79	Willow		Pathogenic	Positive
A. tumefaciens G28/79	Willow	Wisconsin	Pathogenic	Positive
A. radiobacter G42/79	Cottonwood			Negative
A. tumefaciens G44/79	Cottonwood		Pathogenic	Positive
A. radiobacter H7 (2)/80	Rose		Pathogenic	Positive
A. tumefaciens PW4				Positive
A. tumefaciens PW5	Water		Pathogenic	Positive
Agrobacterium J7/79	Apple	Libya		Positive
A. radiobacter K16	Peach		Pathogenic	Positive
A. tumefaciens AR5K/71	Apple		Non-pathogenic	Positive
A. tumefaciens J1/72	Peach		Pathogenic	Positive
A. tumefaciens M4/73	Birch		Pathogenic	Positive
A. tumefaciens C16/80	Apple		Non-pathogenic	Positive
Agrobacterium spp. AE43/96	Grape			Positive
Agrobacterium spp. CP218/95	Grape	California		Positive
A. tumefaciens SOB107	Cherry			Positive
A. tumefaciens ZO9	Pear	Italy		Positive
A. radiobacter ZO33	Cherry	Italy		Positive
A. tumefaciens AA15/96	Viburnum	Pennsylvania	Non-pathogenic	Negative
A. tumefaciens AC27/96	Pieris	Pennsylvania	Non-pathogenic	Negative
A. tumefaciens ARK/71	Crabapple	Washington	Non-pathogenic	Negative
A. tumefaciens AR13K/71	Crabapple	Washington	Pathogenic	Positive
A. tumefaciens B24/93	Quince	Oregon	Non-pathogenic	Positive
A. tumefaciens B49C/83	Apple seedling	Washington	Pathogenic	Positive
A. tumefaciens D10b/87	Mall. Apple	Washington	Pathogenic	Positive
A. tumefaciens F1/75	Boysenberry	Oregon	Pathogenic	Positive
A. tumefaciens F1/79	Baby's Breath	Florida	Non-pathogenic	Negative
A. tumefaciens F8/79	Baby's Breath	Florida	Non-pathogenic	Negative
A. tumefaciens H1/93	Mint	Oregon	Non-pathogenic	Negative
A. tumefaciens H2/72	Azalea		Non-pathogenic	Negative
A. tumefaciens S11/72	Incense Cedar	Oregon	Non-pathogenic	Negative
A. tumefaciens ZO40	Almond		Pathogenic	Positive

Probe design

The capture probe for this study was designed to work with existing PCR primers A and C, which are considered universal for the detection of phytopathogenic *Agrobacterium* and are widely used to screen for the disease in conventional PCR assays (6). Oligonucleotide probe BP-WJT01 was designed to target a region of the *virD2* gene on the virulence plasmid of *Agrobacterium*. We hypothesized that it might be possible to prime directly from the probe, simplifying the PCR assay after the MHC purification. The probe was biotinylated at the 5´ end to allow the immobilization of the target DNA to magnetic beads without interfering with the probe's potential utility as a primer.

Plant inoculations

To generate the symptomatic tissue needed for this study, we prepared bacterial suspensions combining four phytopathogenic strains of *Agrobacterium tumefaciens* in 10mM $MgCl_2$ at a concentration of 100,000 colony forming units per milliliter of liquid (cfu/ml). 'Head Over Heels' rose stems were wounded with a scalpel, and 50 µl of bacterial suspension was applied to each wound. Wounds were subsequently wrapped with Parafilm. For one control group, plants were wounded as described above, and 50 µl of sterile 10mM $MgCl_2$ was applied to the wound before wrapping with Parafilm. A second control group was wounded, and the wounds were wrapped without being treated.

DNA extraction

Approximately 8 weeks after inoculation, tissue samples were collected from treated rose plants. A sterile razor blade was used to collect thin slices of gall (symptomatic) tissue or asymptomatic tissue. Approximately 100 mg of tissue was frozen with liquid nitrogen and pulverized with a sterile mortar and pestle. The macerated plant tissue was transferred to a 2.0-ml Lysing Matrix A tube (MPBio, USA) and homogenized in a FastPrep 24 homogenizer (MPBio, USA) set to 6.0 for 1 min. Subsequently, 400 µl of CTAB buffer amended with 1% polyvinyl pyrrolidone was added to the Lysing Matrix A tube, and the homogenization step was repeated. Total genomic DNA was isolated from the homogenized plant tissue using the DNeasy Plant Minikit (Qiagen, USA) according the manufacturer's protocol. Final concentration of isolated DNA was confirmed using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The isolated DNA was divided into two aliquots, one of which was used for conventional PCR according to existing protocols, and one for further purification using MCH.

Magnetic capture hybridization (MCH)

In magnetic capture hybridization, target DNA is immobilized by magnetic beads conjugated to the oligonucleotide probe that binds to the target region. To prepare the conjugated probe, we added 50 µl of streptavidin-coated magnetic beads (New England Biosciences, USA) to a 1.5-ml microcentrifuge tube and immobilized the beads on a magnetic stand. The storage buffer was removed, and the beads were re-suspended in the same volume (50 µl) of 10µM BP-WJT01 and 1 ml of wash/binding (W/B) buffer (20mM Tris-HCl at pH 7.5, 1mM EDTA, 500mM NaCl). The suspension was incubated at room temperature for one hour with agitation, after which the conjugated beads were immobilized magnetically and the buffer removed. The conjugated beads were re-suspended in 1 ml of denaturation buffer (0.125M NaOH and 0.5M NaCl) and incubated at room temperature for 15 minutes with agitation. The beads were again immobilized on the magnetic stand, the denaturation buffer was

removed, and the beads were washed three times with W/B buffer. Finally, the conjugated beads were re-suspended in their original volume (50 µl) of W/B buffer and stored at 4°C for use within a few days.

For the hybridization procedure, 10 µl of total genomic DNA (200~400 ng of DNA) were added to 1 ml DIG Easy Hyb buffer and incubated at 100°C for 15 minutes. The mixture was then incubated on ice for 2 minutes, after which 20 µl of streptavidin beads conjugated to BP-WJT01 were added with gentle mixing. The mixture was incubated at room temp with agitation for 1 hour, after which the conjugated beads were immobilized magnetically and the buffer removed. The beads were washed three times with sterile, molecular-grade water before re-suspension in 25 µl of sterile water. This mixture was incubated at 95°C for 10 min to disrupt the streptavidin-biotin bond, removing both target DNA and probe BP-WJT01 from the streptavidin-coated beads. Beads were immobilized magnetically, and the remaining solution transferred to a new tube for use in downstream PCR applications.

Results

Oligonucleotide probe BP-WJT01 functions as a universal primer for phytopathogenic *Agrobacterium*. We needed to verify that our oligonucleotide probe would be universal across species, strains, and isolates of *Agrobacterium*. Using conventional PCR, in which BP-WJT01 was partnered with primer A, we screened 44 isolates of *Agrobacterium* representing a wide spectrum of hosts, geographic distribution, species, and strains. Of these strains, 14 had previously yielded negative results in pathogenicity tests. Using this conventional PCR approach, we obtained results that closely matched the results of previous tests. Our probe functioned as an equivalent to primer C, successfully amplifying the target DNA in the pathogenic *Agrobacterium* strains tested. When the probe was used to screen non-pathogenic isolates, amplification of the target DNA was not observed (Figure 1). We therefore determined that BP-WJT01 should be sufficiently sensitive to distinguish between phytopathogenic and non-pathogenic *Agrobacterium*.

MCH improves sensitivity of conventional PCR with a standard Taq DNA polymerase. While our objective was the development of an MCH-real-time PCR assay for the benefit the floral industry, we also hypothesized that the addition of an MCH step prior to conventional PCR could increase the sensitivity of existing assays for laboratory facilities that might not have access to real-time PCR technology. For this procedure, we eliminated the final 95°C incubation step described in the *Magnetic Capture Hybridization* section of the Materials and Methods. This step is designed to remove the probe and captured target DNA from the beads for downstream use; however, we hypothesized that removal of probe and target DNA was unnecessary for conventional PCR, as the reaction could presumably proceed in the presence of the magnetic beads. Therefore, after washing the conjugated beads with sterile water as described previously, we re-suspended the beads in the reaction mix for the conventional PCR assay. Upon completion of the standard PCR protocol, gel electrophoresis was conducted using the reaction mix, still combined with the streptavidin-coated magnetic beads. Using this method, we were able to detect pathogenic *Agrobacterium* from our inoculated rose tissue. Furthermore, adding the MCH purification step to the standard PCR procedure allowed us to detect *Agrobacterium* in samples in which we were unable to detect *Agrobacterium* using conventional PCR without an MCH step (Figure 2).

MCH-RT-PCR detects *Agrobacterium* in rose samples.

We developed our real-time PCR protocol using SYBR Green as a fluorescent reporter of DNA synthesis. SYBR Green is an intercalating agent that binds to all double-stranded DNA, making it less sequence specific than other fluorescent reporter technologies, such as Taqman probes, that require nearly perfect identity between the probe and the sequence of interest in order for binding to occur. While in some cases greater specificity may be desirable, for the purposes of our assay SYBR Green affords greater flexibility, as it will not be affected by differences in the target sequences between strains of *Agrobacterium*. Furthermore, the cost of SYBR Green is significantly less than that of Taqman probes and similar technologies that might make routine screening via real-time PCR cost-prohibitive. One caveat in using SYBR Green as a fluorescent reporter is that it does not differentiate between the target of interest and non-specific products that may be generated during the PCR reaction. Because non-specific amplification, specifically the production of primer dimers, is common in PCR reactions in which the target is not present, the binding of SYBR green to these non-specific products may yield false positives. To differentiate between these false positives and fluorescence generated by amplification of the desired product, a melting curve is added to the end of the thermocycler protocol. For each PCR reaction, the thermocycler measures the temperature at which fluorescence is quenched by dissociation of the double-stranded DNA product. For false positives, such as those generated by primer dimers, the double-stranded DNA products are typically easier to separate, resulting in a lower melting peak than that obtained from the target sequence. By incorporating negative controls, such as a no-template control (water added to the reaction instead of template DNA) and a non-target organism which does not contain the target sequence, we are able to differentiate between true positives and false positives. To test our detection protocol, we conducted magnetic capture hybridization on total genomic DNA collected from symptomatic rose tissue, as well as total genomic DNA isolated from pure culture of a known phytopathogenic *Agrobacterium* strain C58 (positive control) and a non-*Agrobacterium* phytopathogen, *Rhodococcus fascians* A44a (negative control). A no-template control consisted of a water-only sample subjected to all of the steps of MCH and real-time PCR. Using this method, we were able to detect *Agrobacterium* in infected rose tissue in multiple experiments. In these experiments, the melting peak obtained from the infected rose sample closely matched that of the positive control *A. tumefaciens* C58 (Figure 3). In some cases, either the negative control or no-template control emitted sufficient fluorescence to register as a positive result, but in most cases, the product was too weakly associated or lacked sufficient abundance to yield a melting peak (Figure 3). The lack of a melting peak or, in some cases, a melting peak much lower than that of the positive control and the sample, indicates that false positives can be distinguished from true positives.

Inhibitor-resistant Taq DNA polymerase provides superior results to MCH.

The primary difficulty we encountered with the MCH technique was in recovering sufficient quantities of DNA from the beads in the elution step. After boiling the conjugated samples to dissociate the probe-target DNA complex from the streptavidin coated beads, we consistently obtained a low yield of total DNA, often <10 ng/ml. As a result of the low concentrations of DNA obtained via MCH, we found our MCH-assisted real-time PCR screen to less reliable than desired, resulting in frequent false negatives. As an alternative, we examined the utility of using an inhibitor-resistant Taq DNA polymerase mix to

interrogate total genomic DNA from plant tissue without the use of MCH. We found that conventional PCR using the inhibitor-resistant DNA polymerase mix yielded results superior in accuracy and consistency to those obtained from both MCH real-time PCR and conventional PCR using bead-bound DNA as a template (Figure 4).

Inhibitor-resistant Taq DNA polymerase detects *Agrobacterium* in symptomatic and asymptomatic tissues.

The conventional PCR screen using Accustart II PCR ToughMix as the DNA polymerase provided robust detection of *Agrobacterium* in gall tissue. The pathogen was detected in all 20 of the samples collected from symptomatic roses across multiple replicates of the experiment. In one replicate, *Agrobacterium* was also detected in asymptomatic tissue collected from a plant that had been wounded but not inoculated as a negative control (Fig. 5A). Although all of the experimental plants were determined to be disease free prior to the experiment, this determination was made using conventional PCR with standard Taq DNA polymerase, a screening method which we have shown to be less robust than the method we used in the experiment. The greater sensitivity of the PCR screen using the inhibitor-resistant DNA polymerase may have detected a low level of *Agrobacterium* infection in the asymptomatic tissue that was not detectable by the earlier method. Moreover, when we tested asymptomatic tissue from twig tissue and root tissue using the improved, inhibitor-resistant method, we detected *Agrobacterium* in 50-60% of the inoculated samples, and in 20-50% of our negative control samples in multiple replicates. This would seem to support our hypothesis that the improved PCR detected low levels of *Agrobacterium* infection in plants that had previously tested negative.

Conclusions

Magnetic capture hybridization is a technique that is commonly used to separate genomic DNA from PCR inhibitors present in soil, plant tissue, and other matrices with complex chemistry. In this study, we have demonstrated that MCH can be successfully used to purify total genomic DNA from symptomatic tissue of rose, a plant type that has historically been recalcitrant to PCR, making diagnosis of crown gall disease problematic. The probe we designed for this study, BP-WJT01, has been shown to function as a universal primer for phytopathogenic *Agrobacterium* with efficacy equal to that of existing universal primers. Our screens of 44 isolates of *Agrobacterium* from disparate geographical regions and hosts demonstrated the robustness of our probe in distinguishing between pathogenic and non-pathogenic isolates of multiple species.

While the purification of total genomic DNA from symptomatic plant tissue using MCH improves the sensitivity of conventional PCR, we found that the same benefit could be obtained more reliably and simply by using an improved, inhibitor-resistant formulation of Taq DNA polymerase. While this improved DNA polymerase is not yet available as part of a real-time PCR mix, in our hands it has made conventional PCR using traditional AC primers into a reliable, accurate method of screening for *Agrobacterium* in rose tissue. Nevertheless, the demonstrated utility of the probe we designed for this study means that it could be used in situations where RT-PCR is required, or when purification of target DNA for downstream applications other than PCR is desired.

The results we obtained from screening non-gall tissue in this study underline the need for a more accurate PCR screen for *Agrobacterium*, such as the one we have described here. While initial PCR screens of experimental plants using conventional PCR with standard Taq DNA polymerase indicated

that they were free of *Agrobacterium*, our improved PCR screen detected the pathogen in untreated plants as well as plants that were inoculated with *Agrobacterium*. Thus, although we detected *Agrobacterium* in twig and root tissue distal from the site of inoculation in our *Agrobacterium*-treated plants in 50-60% of the samples tested, we cannot draw meaningful conclusions about the implications for movement of the pathogen through the plant, as the detection rate was only slightly lower in the untreated plants.

Plant-derived compounds that inhibit PCR are the primary obstacle to reliably accurate detection of crown gall-causing *Agrobacterium* in woody plants. We have shown that an inhibitor-resistant DNA polymerase can make conventional PCR a reliable method for screening for this organism. Development of a real-time PCR chemistry that includes the improved DNA polymerase is a logical next step in the improvement of this assay. Furthermore, we have demonstrated that the biotinylated probe developed for this study is a versatile tool for the selective purification of target DNA from many virulent strains across multiple species of *Agrobacterium*. With regard to our screening of non-gall plant tissues, we have shown that improved PCR can detect pathogenic *Agrobacterium* in asymptomatic tissue. Moreover, our results indicate that the pathogen may be lurking in asymptomatic tissues at levels too low to detect by methods currently in use.

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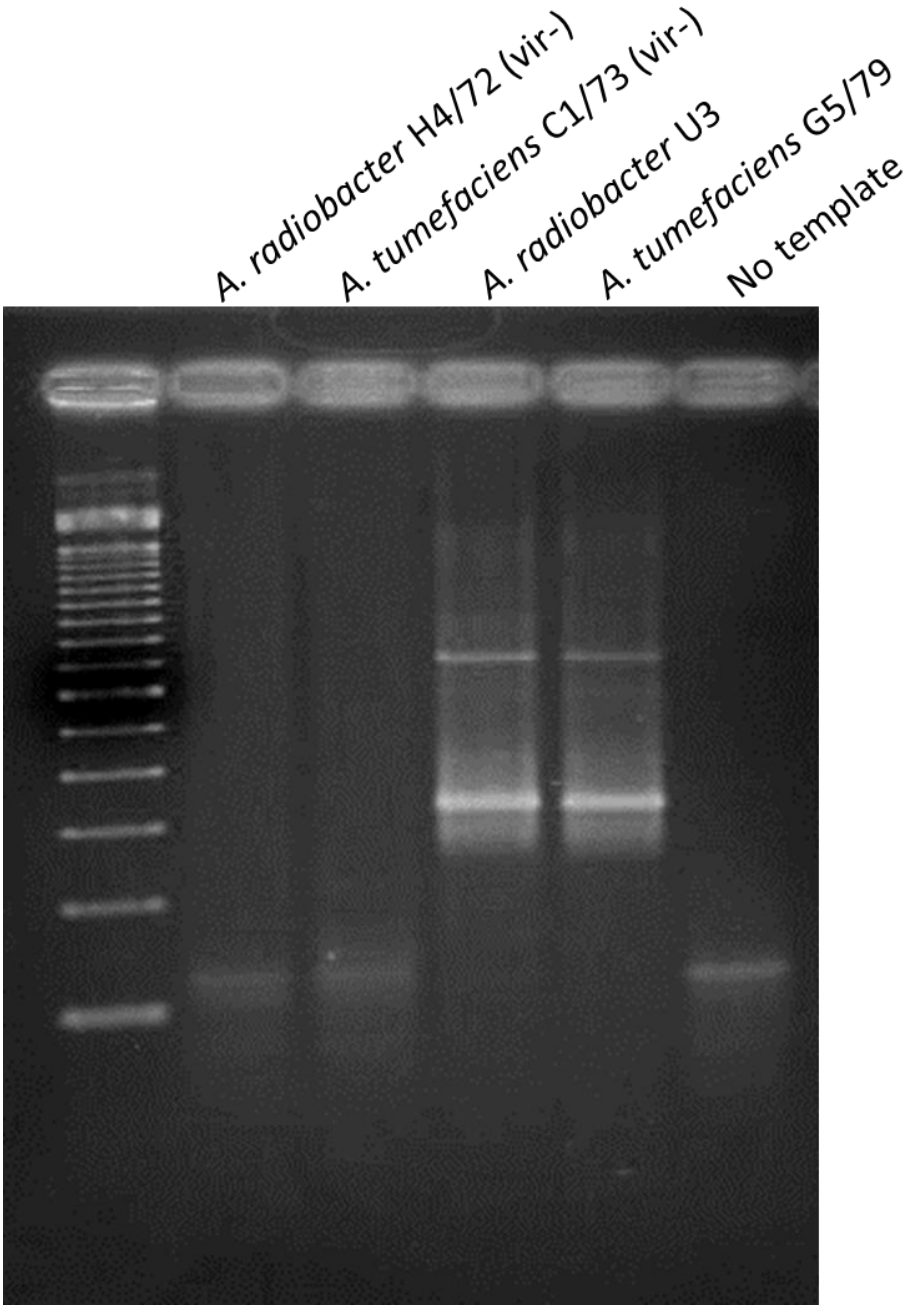


Figure 1. Oligonucleotide probe BP-WJT01 functions as a universal primer for phytopathogenic *Agrobacterium*. Two strains of non-pathogenic *Agrobacterium* (designated vir-) and two strains of phytopathogenic *Agrobacterium* were screened using conventional PCR with primer A (Haas, 1995) and BP-WJT01. The expected product of ~325 bp was amplified from the known phytopathogens, while the non-pathogenic strains yielded results similar to the no-template control. Of the 49 strains of *Agrobacterium* that were screened for this study, all 37 of the known phytopathogens screened positive using conventional PCR. Similarly, all 12 of the known non-pathogenic strains yielded negative results using this method.

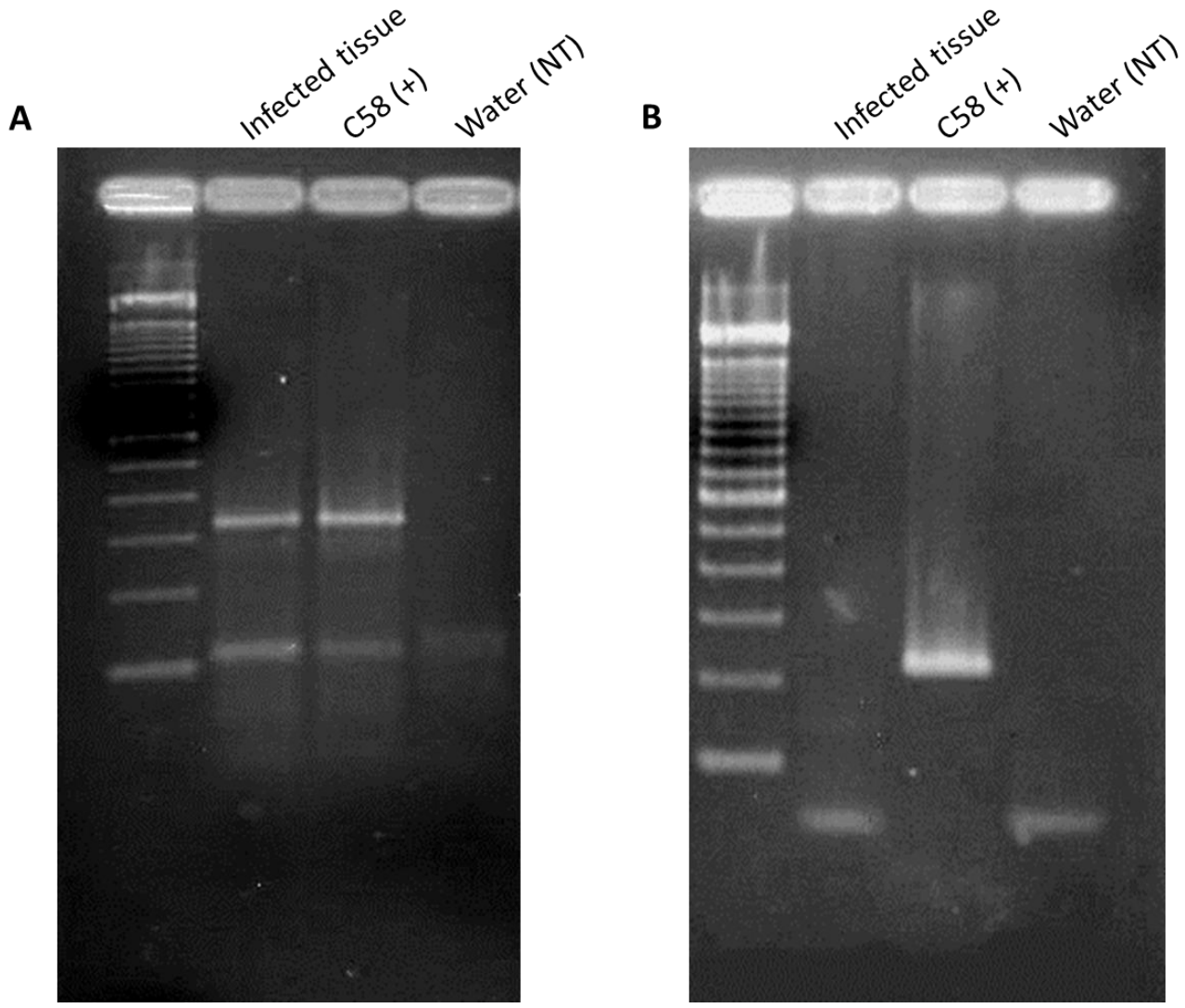
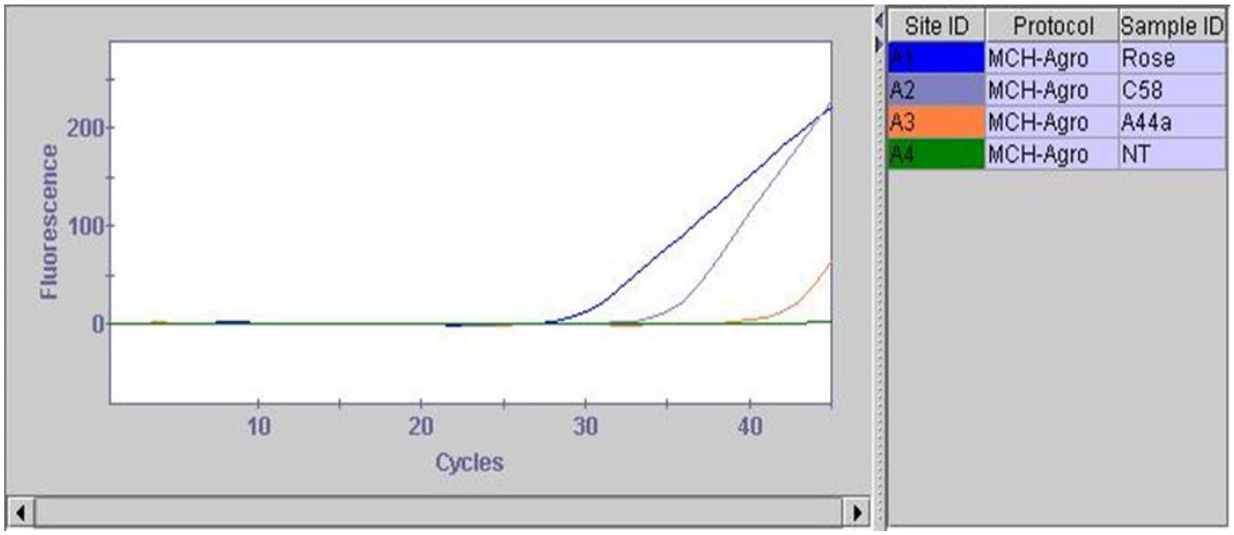


Figure 2. MCH improves sensitivity of conventional PCR using standard Taq DNA polymerase. A) Conventional PCR assay detects *Agrobacterium* in total genomic DNA from symptomatic rose tissue after MCH purification. B) Conventional PCR without MCH does not detect *Agrobacterium* in the same sample.

A

Sample ID	Result	Intcltr C_t	Melt Peak1
Rose	POS	31.69	83.26
C58	POS	36.43	84.46
A44a	POS	43.39	
NT	NEG	0	

B



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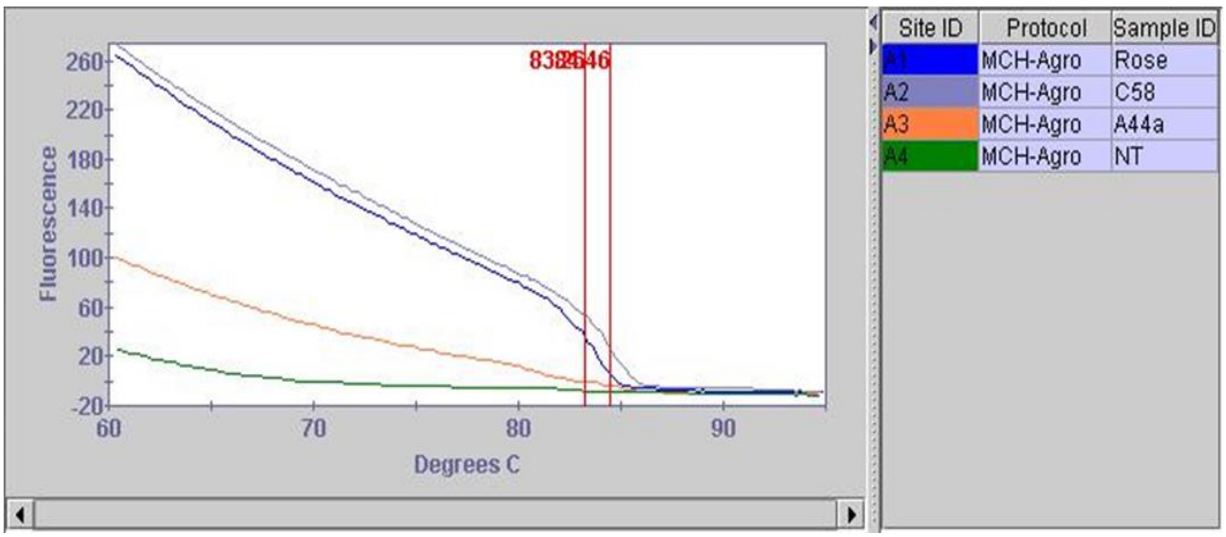
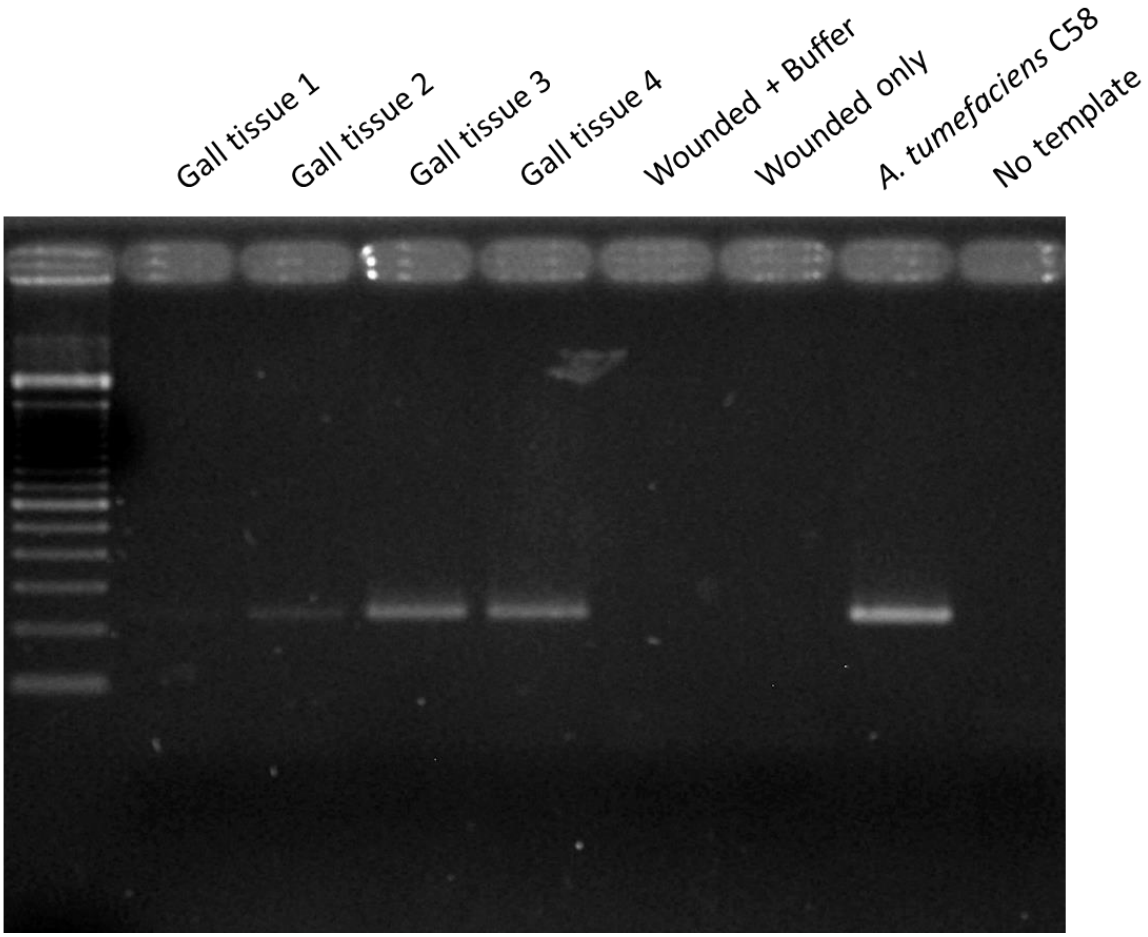
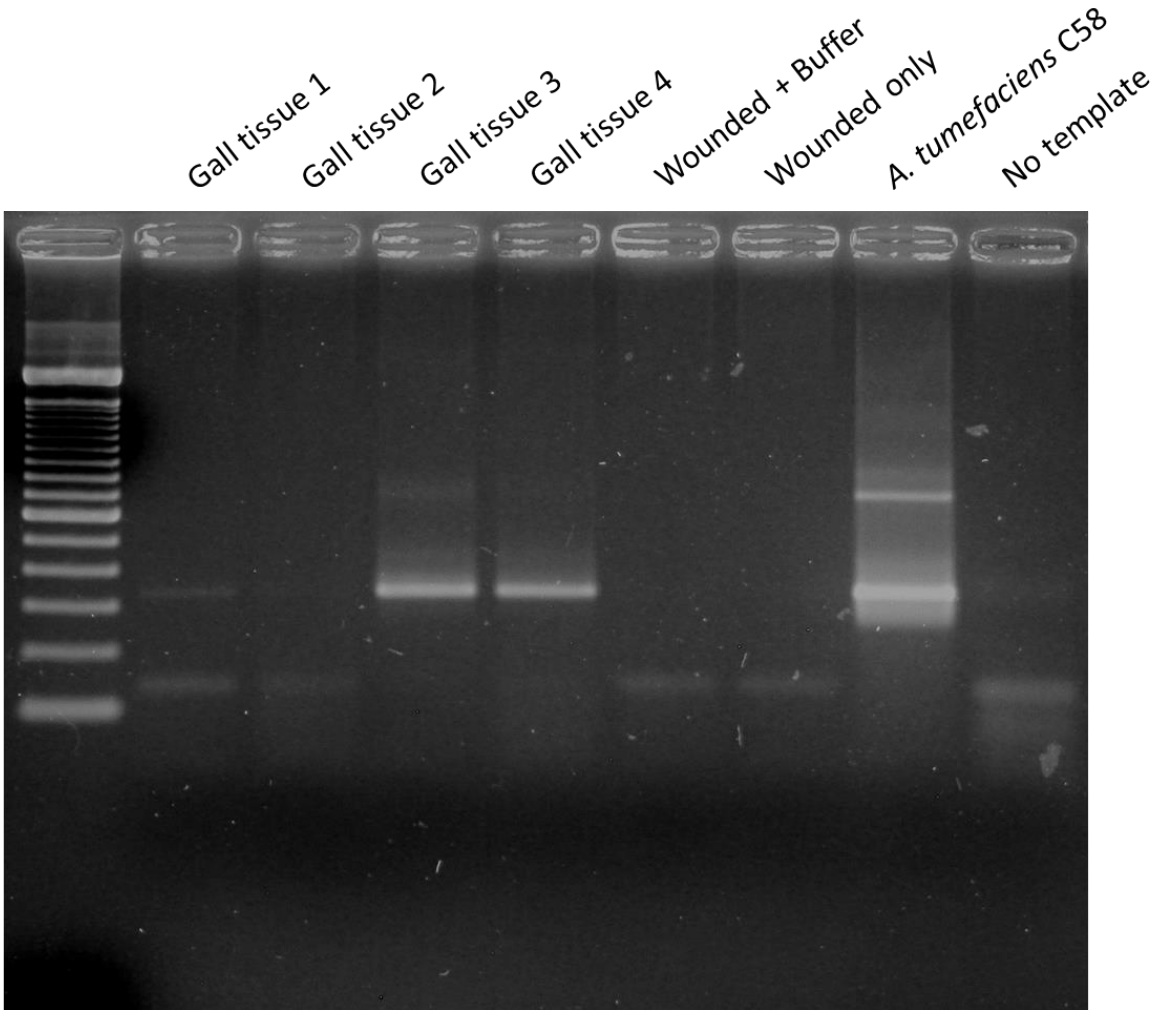


Figure 3. MCH-RT-PCR detects phytopathogenic *Agrobacterium* in rose samples. A) Abridged output of Cepheid SmartCycler software, showing result, C_t (the cycle at which fluorescence passed the threshold of detection) and melting peak, indicating the temperature at which the single-stranded DNA dissociates, quenching fluorescence. B) Graphical representation of the data from the table, also output by the Cepheid SmartCycler software. C) Graph of melt curve, indicating how melting peaks of PCR products are determined. The melt curve is used to differentiate between fluorescence caused by a positive result and fluorescence resulting from primer dimers, which may yield false positives. In the figure above, the data in A) and B) indicate that the negative control, A44a, yielded sufficient fluorescence to register a positive result; however, the melt curve data from A) and C) indicate that the double-stranded DNA responsible for the fluorescence was too weakly associated to yield a melting peak; therefore, we determine that the result for A44a is, in fact, negative. This experiment was repeated three times with similar results.

A


Gall tissue 1
Gall tissue 2
Gall tissue 3
Gall tissue 4
Wounded + Buffer
Wounded only
A. tumefaciens C58
No template

B


Gall tissue 1
Gall tissue 2
Gall tissue 3
Gall tissue 4
Wounded + Buffer
Wounded only
A. tumefaciens C58
No template

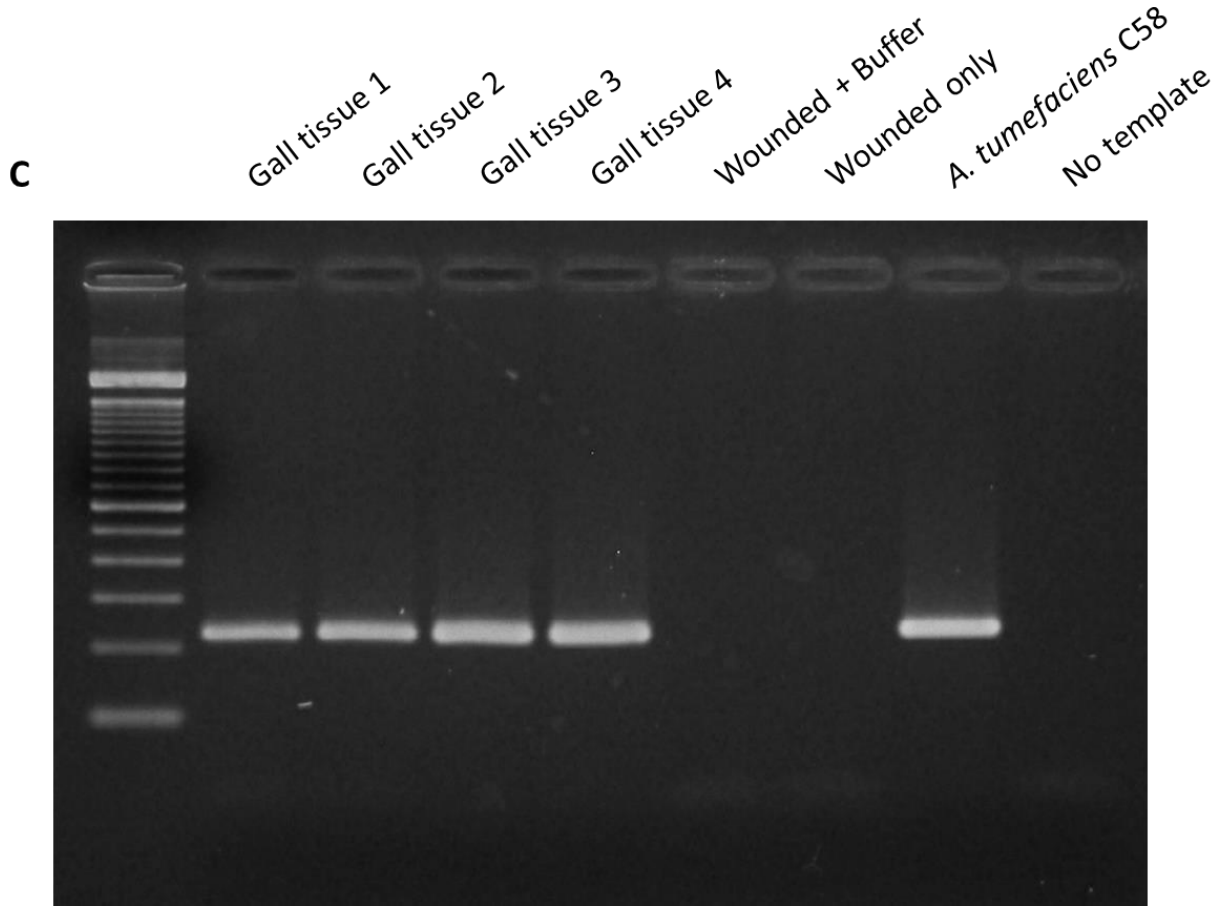
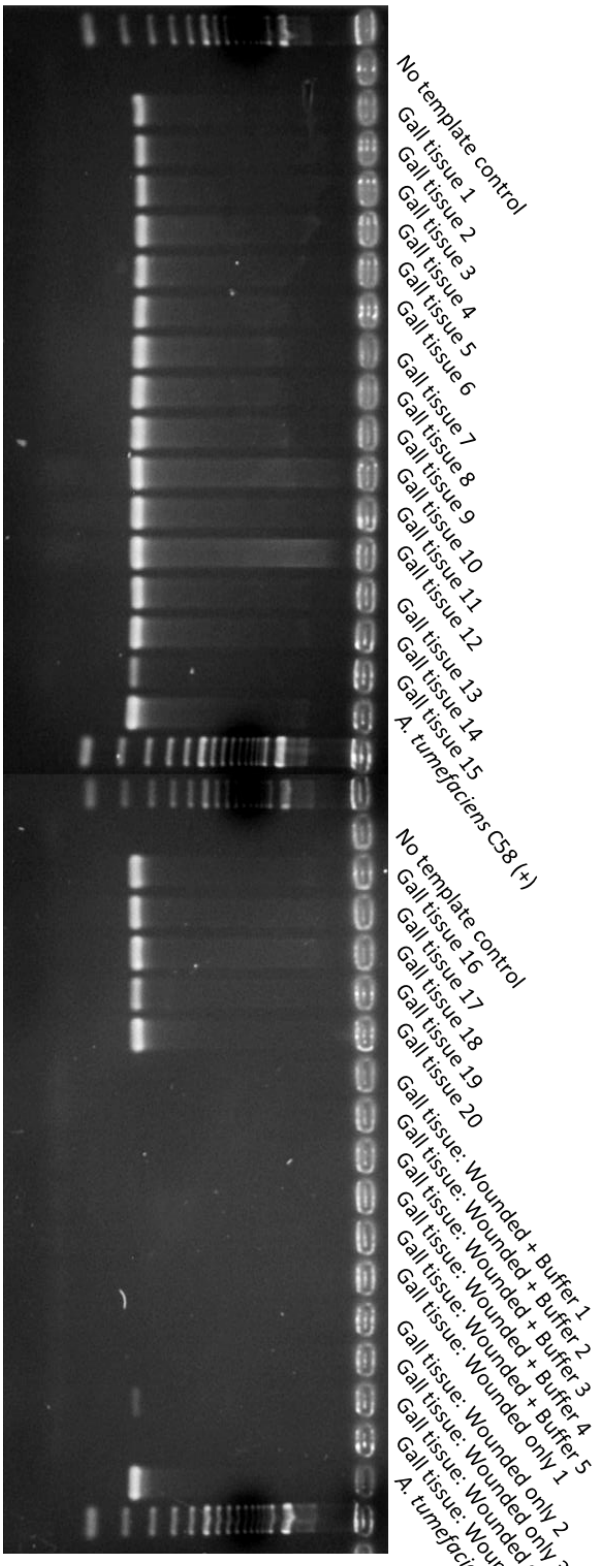
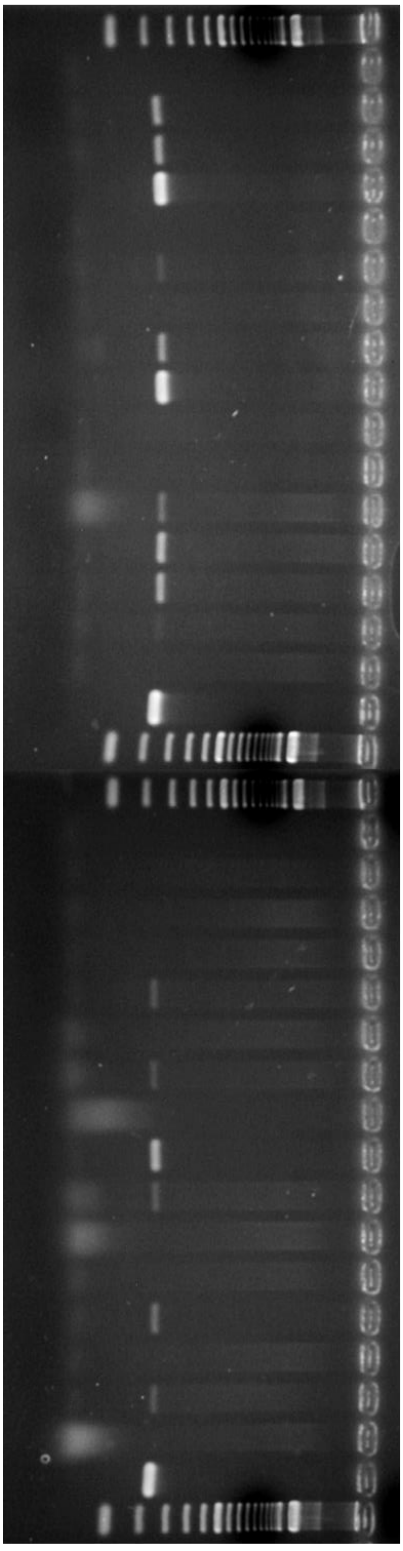


Figure 4. Inhibitor-resistant Taq DNA polymerase provides superior results to MCH. A) Results of a conventional PCR using total genomic plant DNA from four different galls as a template. A standard Taq DNA polymerase (New England Biosciences) was used. B) The same conventional PCR screen was repeated with a template consisting of streptavidin-coated beads conjugated to capture probe BP-WJT01 and hybridized to target DNA. C) The conventional PCR screen was repeated again, replacing the standard Taq DNA polymerase with inhibitor-resistant Accustart II ToughMix (Quanta). *A. tumefaciens* was strongly detected in all samples under these conditions.


A
No template control
Gall tissue 1
Gall tissue 2
Gall tissue 3
Gall tissue 4
Gall tissue 5
Gall tissue 6
Gall tissue 7
Gall tissue 8
Gall tissue 9
Gall tissue 10
Gall tissue 11
Gall tissue 12
Gall tissue 13
Gall tissue 14
Gall tissue 15
A. tumefaciens C58 (+)
No template control
Gall tissue 16
Gall tissue 17
Gall tissue 18
Gall tissue 19
Gall tissue 20
Gall tissue: Wounded + Buffer 1
Gall tissue: Wounded + Buffer 2
Gall tissue: Wounded + Buffer 3
Gall tissue: Wounded + Buffer 4
Gall tissue: Wounded + Buffer 5
Gall tissue: Wounded only 1
Gall tissue: Wounded only 2
Gall tissue: Wounded only 3
Gall tissue: Wounded only 4
Gall tissue: Wounded only 5
A. tumefaciens C58 (+)

B



No template control
Twig tissue 1
Twig tissue 2
Twig tissue 3
Twig tissue 4
Twig tissue 5
Twig tissue 6
Twig tissue 7
Twig tissue 8
Twig tissue 9
Twig tissue 10
Twig tissue 11
Twig tissue 12
Twig tissue 13
Twig tissue 14
Twig tissue 15
A. tumefaciens C58 (+)
No template control
Twig tissue 16
Twig tissue 17
Twig tissue 18
Twig tissue 19
Twig tissue 20
Twig tissue: Wounded + Buffer 1
Twig tissue: Wounded + Buffer 2
Twig tissue: Wounded + Buffer 3
Twig tissue: Wounded + Buffer 4
Twig tissue: Wounded + Buffer 5
Twig tissue: Wounded only 1
Twig tissue: Wounded only 2
Twig tissue: Wounded only 3
Twig tissue: Wounded only 4
Twig tissue: Wounded only 5
A. tumefaciens C58 (+)

C

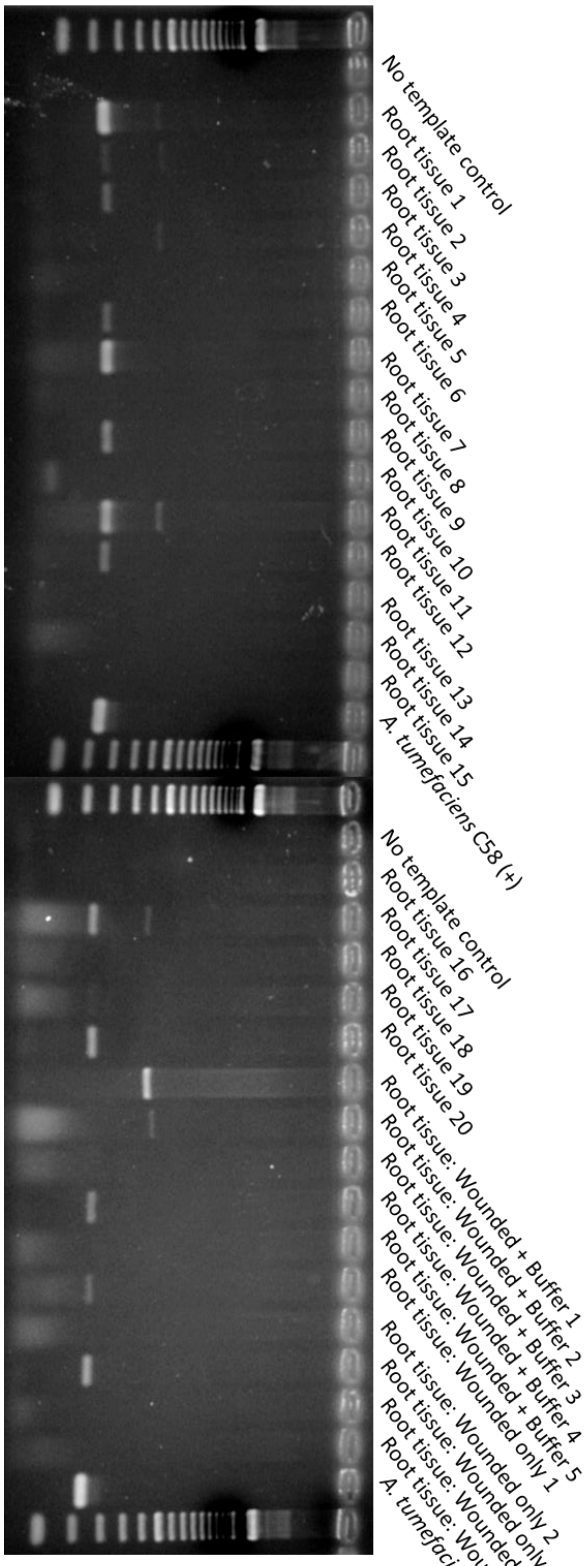

No template control
Root tissue 1
Root tissue 2
Root tissue 3
Root tissue 4
Root tissue 5
Root tissue 6
Root tissue 7
Root tissue 8
Root tissue 9
Root tissue 10
Root tissue 11
Root tissue 12
Root tissue 13
Root tissue 14
Root tissue 15
A. tumefaciens C58 (+)
No template control
Root tissue 16
Root tissue 17
Root tissue 18
Root tissue 19
Root tissue 20
Root tissue: Wounded + Buffer 1
Root tissue: Wounded + Buffer 2
Root tissue: Wounded + Buffer 3
Root tissue: Wounded + Buffer 4
Root tissue: Wounded + Buffer 5
Root tissue: Wounded only 1
Root tissue: Wounded only 2
Root tissue: Wounded only 3
Root tissue: Wounded only 4
Root tissue: Wounded only 5
A. tumefaciens C58 (+)

Figure 5. Inhibitor-resistant Taq polymerase detects *Agrobacterium* in symptomatic and asymptomatic tissues. A) *Agrobacterium* was detected in total genomic DNA collected from symptomatic tissues using the CTAB extraction method and screened via conventional PCR using the Accustart II PCR ToughMix. *Agrobacterium* was also weakly detected in one of the negative control samples, consisting of asymptomatic tissue from a wound site. B) *Agrobacterium* was detected in 11 of the 20 samples of asymptomatic twig tissue collected from inoculated plants. However, *Agrobacterium* was also detected in 5 out of 10 of the negative control samples, which consisted of asymptomatic tissue collected from plants that were wounded but not inoculated with bacteria. C) *Agrobacterium* was detected in 10 of the 20 samples of asymptomatic root tissue collected from inoculated plants. *Agrobacterium* was also detected in 3 of the 10 samples of root tissue collected from uninoculated plants. Similar results were obtained from multiple replicates of these experiments.