Detection and Differentiation of Pathogenic *Agrobacterium vitis* and *A. tumefaciens* in Grapevine using Multiplex Bio-PCR

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Crown gall caused by *Agrobacterium vitis and A. tumefaciens* occurs in grape growing areas worldwide. Infected vines can harbor both pathogenic and nonpathogenic strains and remain symptomless until the vines are injured (Burr and Katz 1983, 1984). Injury can cause galls which interfere with the function of the vascular system of the plant and reduce its vigor and productivity. To limit the spread of crown gall in vineyards, it is important to test propagation materials and ensure they are free from pathogenic *Agrobacterium* prior to planting. Polymerase chain reaction (PCR) is quickly replacing many of the slower traditional methods of diagnosing *Agrobacterium*; however, many existing primers have limitations due to the genetic diversity of the *Agrobacterium* spp. For example, some primers that can detect pathogenic *A. tumefaciens* fail to consistently detect pathogenic *A. vitis*. Other primers are limited to detecting only specific opine types of *A. vitis* and *A. tumefaciens*. Furthermore, certain primers fail to distinguish between the virulent and avirulent strains and some give amplifications that are not reproducible or are non-specific.

The purpose of this study was to compare available *Agrobacterium* primer sets and identify a more reliable PCR test that can detect pathogenic *A. vitis* and *A. tumefaciens* in grapevine and differentiate between the two species. The specificity and reproducibility of several primers in detecting pathogenic strains were tested using colony and Bio-PCR. Virulence specific universal primers were selected in this study to detect the *vir*D2, *vir*C and *vir*F regions of the Ti plasmid. The primer sets virD2A/2C (Haas *et al*. 1995) and VCF3/VCR3 (Suzaki *et al*. 2004) were used to detect the *vir*D2 and the *vir*C genes, respectively. A combination of *vir*FF1/*vir*FR2 and *vir*D2S4F/*vir*D2S4R (Bini *et al*. in press) was used in a multiplex PCR to detect the *vir*F and *vir*D2 regions in the most common opine types of *A. vitis* which are nopaline, octopine and vitopine types (Burr *et al*. 1998, Ride *et al*. 2000). In order to identify *A. vitis* strains and differentiate them from the *A. tumefaciens* strains, PGF/PGR (Szegedi and Bottka, 2002), a polygalacturonase specific primer set was used. Twenty-two strains and 17 Bio-PCR preps were included in this study. The pathogenicity of the strains was confirmed on tomatoes, tobacco and carrot disks.

Materials and Methods

Twenty-two young, randomly selected rooted benchgrafts from various California nurseries and one older vine from Virginia were tested. Out of 22 samples, 6 were symptomatic and 16 were asymptomatic. Fifteen bacterial strains isolated from the samples and seven reference strains from Dr. Burr were tested. Bacteria were extracted from vascular tissue of galls, crown, roots and canes that were suspended in sterile dH_2O for 1 hour at room temperature. A dilution series of 10^0–10^{-3} were made from the extract and 100µl of each dilution was plated on RS agar plates (Moore *et al*. 2001). The plates were incubated at 27°C. After four days, suspect colonies were subcultured and purified on Potato dextrose agar containing 0.5% calcium carbonate (Moore *et al*. 2001). For the single colony PCR reactions, one loopful of a 24-48 h culture suspended in 1 ml of extraction buffer (Osman and Rowhani, 2006) was used as a template. Template for the Bio-PCR reactions was made by suspending four day old growth on RS plates in 2 mls of extraction buffer. For the PCR reaction, 2 µl of template was used in a 25 µl reaction for PCR amplification and combined with 1X PCR buffer (20mM Tris-Cl, pH 8.4, 50mM KCL), 1X sucrose red dye solution (2% sucrose, 0.1mM crysol red), 0.5 µM of both forward and reverse primers, 5 mM DTT, 1.5mM MgCl2, 0.2 mM dNTPs, 0.5U Taq polymerase. The amplification was started with an initial denaturation step at 94°C, 5 min; followed by 35 cycles of (94°C for 1 min, primer-specific annealing temperature for 1 min, 72°C for 1 min); with a final extension at 72°C for 5 min. An annealing temperature of 43°C was used for virD2A/2C, 56°C for VCF3/R3 and PGF/R, and 60°C for the opine primers. PCR products were separated on 1.8% agarose gel via electrophoresis, stained with ethidium bromide and observed under UV. Strains were inoculated on tomato, tobacco or carrot disks to confirm their pathogenicity.

Results and Discussion

Based on this study, the new primer set VCF3/VCR3 developed by Suzaki *et al*. proved to be the most reliable in detecting the pathogenic strains of *A. vitis* and *A. tumefaciens* in grapevine. VCF3/VCR3 detected all the pathogenic strains in this study, whereas, virFF1/virFR2 and virD2S4R/virD2S4F failed to detect two *A. vitis* strains. Since the virFF1/virFR2 and virD2S4R/virD2S4F primers were made specifically to detect the octopine, nopaline, and vitopine types of *A. vitis*, the two strains that were not detected may be of variant opine type pointing out the possible limitations of these primer sets. VirD2A/virD2C primers consistently detected pathogenic *A. tumefaciens* but not the pathogenic *A. vitis* strains. VirD2A/virD2C failed to detect one pathogenic *A. vitis* strain and produced weak signals with two other *A. vitis* strains even under a low annealing temperature of 43°C. Similar findings were reported by Bini *et al*. They later showed that there are two different nucleotide sequences of the virD2 in *A. vitis* which may contribute to the inconsistencies of this primer set in detecting pathogenic *A. vitis* strains (Bini *et al.*, in press). PGF/PGR primers detected both pathogenic and non-pathogenic strains of *A. vitis* but did not detect *A. tumefaciens* strains as expected.

Although the previous work done with the VCF3/VCR3 primers involved only single colony PCR of *Agrobacterium* strains isolated from infected apple saplings, we found that this primer set worked just as efficiently in detecting pathogenic *Agrobacterium* strains in grapevine using Bio-PCR, thereby eliminating the tedious step of subculturing and isolation of bacterial colonies. We found 100% correlation between the Bio-PCR and the corresponding single colony PCR results with VCF3/VCR3 primers. Furthermore, we discovered that by combining the PGF/PGR primers with the VCF3/VCR3, we were able to detect both pathogenic *A. vitis* and *A. tumefaciens* and differentiate between the two species in one multiplex Bio-PCR reaction. All the pathogenic *A. vitis* strains produced two bands (466 bp and 414 bp) whereas, all pathogenic *A. tumefaciens* strains produced only one band of 414 bp size. The pathogenicity of all the VCF3/VCR3 positive strains and some negative strains was confirmed on tomato, tobacco or carrot disks resulting in 100% correlation with the PCR results. Based on this preliminary study, the PGF/PGR, VCF3/VCR3 multiplex Bio-PCR is a faster and more sensitive assay that can be a valuable tool for large-scale diagnosis of crown gall in grapevine.

Acknowledgement

Reference strains were kindly provided by Dr.Thomas Burr.

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