2002 EXECUTIVE SUMMARY REPORT

PROJECT TITLE: THE DEVELOPMENT OF A RAPID PCR-BASED METHOD FOR QUANTIFYING BACTERIAL DISEASE ON TOMATO AND PEPPER PLUG SEEDLINGS

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PROJECT OBJECTIVE: To develop a multiplex, real-time fluorogenic PCR-based assay for quantifying bacterial pathogens on greenhouse plug seedlings.

(With a rapid, efficient and reliable means of quantifying bacterial pathogens on symptomless tomato and pepper plug plants we will be able to adequately assess the severity of the disease threat presented by this inoculum source. If we find a significant population of these pathogens on a batch the plug plants, then a potentially serious disease threat and crop loss could be averted by either spraying or destroying the affected material.)

METHODOLOGY:

Tomato leaf processing and DNA extraction. Four-week-old tomato plants (cv. Bonny Best) were sprayed with the bacterial spot pathogen *X. campestris* pv. *vesicatoria* DC93-1 at various concentrations and incubated in the plant growth chamber for 20 hours. Leaf samples (1 g) were taken from the plants, weighed and then washed in sterile water for 30 min. The bacteria were recovered from the wash water by centrifugation. Bacterial DNA was extracted from the pellet by the freeze-boil method, the Qiagen DNeasy kit or the Epicentre MasterPure™ kit. The negative control consisted of wash water with no added bacteria.

Real-time quantitative PCR. The Roche LightCycler Instrument and the Roche FastStart DNA Master SYBR Green I kit were used to perform a quantitative real-time polymerase chain reaction (PCR). Using the Roche LightCycler Probe Design Software, bacterial spot and bacterial speck primers were designed for use in the LightCycler. Each PCR was performed in a 20-µl volume (1.6 µl of 3 mM $MgCl_2$, 1 µl primer mix, 2 µl SYBR green mix, 14.4 µl water and 1 µl DNA template) in a capillary tube. The amplification program began with 10 min at 95 C followed by 37 cycles (ramp rate of 20 C/sec) of 95 C for 0 sec, 60 C (annealing temperature) for 10 sec and 72 C for 25 sec. Standard curves were prepared using kit-purified genomic DNA from the bacterial spot strain DC93-1. Standard curves also were prepared using freeze-boiled extractions of diluted bacterial suspensions. The threshold cycle, C_T, is the cycle number at which fluorescence emission crosses an arbitrarily defined threshold within the logarithmic increase phase. By comparing a sample's C_T value with that found on a standard curve of C_T vs. log concentration, one can determine the amount of cells or DNA in that sample.

RESULTS:

Primer design. PCR primers are short strands of DNA that are required to initiate DNA by the Taq DNA polymerase in a polymerase chain reaction. To ensure that optimal primers were being used in the LightCycler quantitative PCR assay, the sequence of the highly-specific bacterial spot probe

BSX was submitted to analysis using the Roche LightCycler Probe design software. The AAFC-BSX primers, which have been used extensively in qualitative PCR by our laboratory, were not suitable for quantitative PCR in the LightCycler and thus we generated a new set: AAFC-BSX7/8. The amplified fragment produced with AAFC-BSX7/8 primers was 585-bp. In a similar fashion bacterial speck primers for quantitative PCR were designed based on the DNA probe COR15; they were called AAFC-COR7/8.

PCR reaction optimization. The effect of magnesium concentration on the AAFC BSX7/8-based LightCycler reaction was determined. The $MgCl_2$ concentration range used was 1.5, 2, 2.5, 3, 4 and 5 mM. Optimum fluorescent signal without sacrificing specificity was obtained at 2.5 and 3 mM $MgCl_2$. Two different annealing temperatures were examined: 60 and 63 C. The reaction efficiency at the two temperatures was equivalent.

Standard curves. The suitability of the DNA obtained using the various extraction protocols for quantitative PCR was determined using the SYBR Green I protocol. A bacterial suspension was serially diluted and the DNA was extracted from each dilution by the freeze-boil method. This DNA was subjected to PCR in the LightCycler. We then prepared a standard curve in which we measured fluorescence intensity vs. cell number (colony forming units, CFU). The correlation between cell number and fluorescence was good (square regression coefficient $r^2 = 0.92$) but not as linear as that obtained if we first purified the bacterial DNA template ($r^2 = 0.99$). The difference in amplification efficiency between crude extract (freeze-boil) and purified DNA was significant (-4.67 vs. -3.13).

Quantification of bacterial spot on leaf surfaces using the LightCycler. Bacteria were washed off the surfaces of tomato leaves 20 hours after the plants had been sprayed with various concentrations of the bacterial spot strain DC93-1. DNA was extracted from the wash water by either the Qiagen DNeasy kit or the Epicentre MasterPure kit and then subjected to quantitative PCR using the SYBR Green I protocol. With this protocol the presence of primer-dimers reduced the sensitivity of the assay, and thus the detection limit was approximately 4000 bacteria per PCR. Fluorescence emission with lower numbers of bacteria did not differ significantly from that of the water control.

SUMMARY AND CONCLUSIONS

We have developed a real-time quantitative PCR protocol for determining population levels of the bacterial spot pathogen on the leaves of greenhouse grown tomato plants. A standard PCR diagnostic assay, which gives a qualitative result ('yes' or 'no'), is done in a day; the quantitative LightCycler PCR assay, which gives actual numbers of bacteria/gram of leaves, is complete within two hours. Although the FastStart DNA Master SYBR Green I kit used in these experiments minimizes low melting temperature products (such as primer-dimers), there are still sufficient quantities of these products present to reduce the sensitivity of the quantification assay. Our current detection limit is approximately 4000 bacterial cells/reaction. To improve upon the sensitivity, we plan to replace the SYBR Green I protocol with one that uses fluorogenic sequence-specific hybridization probes. Our ultimate goal is to be able to detect 50 or less pathogenic bacteria in a quantitative PCR assay.