Real-time PCR assays based on the multi-copy rDNA ITS region and the single-copy β-tubulin gene for detection and quantification of the strawberry pathogen *Colletotrichum acutatum*.

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Introduction

Colletotrichum acutatum is one of the most important fungal pathogens of strawberry worldwide. In our conditions, disease is mostly expressed as strawberry fruit rot. However, *C. acutatum* may also persist on young strawberry plants without causing visible symptoms. Such latent infections are considered to be the main cause of dissemination of *C. acutatum*. Detection and quantification of *C. acutatum* during this latent phase using real-time PCR might aid considerably in the reduction of its spread.

Brown lesions typical of strawberry fruit rot caused by *C. acutatum*

Objectives

- to develop real-time PCR assays using primers designed to the multi-copy rDNA ITS1 region and the single-copy β-tubulin 2 gene of *C. acutatum*.
- to compare the sensitivity of both assays and to use these data to calculate the genome size and the ITS copy number of *C. acutatum*.
- to detect and quantify *C. acutatum* in infected strawberry leaves.

Methods used

Development and sensitivity of the real-time PCR

The rDNA ITS region and the variable region 1 of the β-tubulin 2 gene of *Colletotrichum* isolates were PCR-amplified, purified and cloned into plasmid vectors. Primers were designed to specifically detect *C. acutatum* (**Figure 1**). Sensitivity and specificity of the primers was evaluated through amplification and melting curve analysis using SYBRGreen or TaqMan technology. Quantification was done using the standard curve technique.

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A
18S                                                ITS1
GATCATTACTGAGTTACCGCTCTATAACCCTTTGTGAACATACCTA--ACCGTTCGGCGGGCAGGGGAAGCCTCTCGCGGGC C.acutatum A2(AF411765)
.....................................G.....--.....................................  A3(AJ536214)
.........................C.....................--.....................T....  A4(AF411766)
.........................C.....................--.....................................  A5(AJ536206)
.....................................................--.....................................  A7(AJ536212)
.........................C.....................--.....................................  A9(DQ991745)
...............TA.............................TA..T..........T.----------....A..C.- C.gloeosporioides (AF411774)
...............TA.......C.....................CA..T..........T----------..C.A..T.- C.fragariae (AF411767)
B
Exon 5                          Intron                                  Exon 6
CGTCTACTTCAACGAAGTTTGTTATCCTAGT-CCCCCAGTGTGCAGGC-AATCCTATTGACGAATGCTGACCTTCTCACCCAACCAGGCCTC C.acutatum A2 (AJ409296)
.....................................................................................  A3 (AJ311668)
.................................T.............................A.....................  A4 (AJ409294)
.....................................G..............C.....................................  A7 (EU635504)
.....................................................................A.....................  A9 (EU635505)
.........................C.T..TAG....A..A.....A.TA..A.A.........G.A.........G.T..T.C......T.. C.gloeosporioides (AJ314714)
......T.................C.T..TAG.......A.....A..A..A.A.........G.A.........G.T..T.C......T.. C.fragariae (EU635506)
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Figure 1. Partial sequence alignment of the 18S-ITS1 region (A) and the β-tubulin 2 variable region 1 (B), showing the position of the primers (arrows) and probe (line) designed for the detection of *C. acutatum* by real-time PCR. Only polymorphic nucleotides are shown. - indicates a gap in the sequence.

Estimation of the genome size and ITS copy number (based on Lee et al., 2008)

The genome size (GS) of *C. acutatum* was estimated using following equations:

$$\text{Mass single genome} = 10^{X_1 - [b_1X_1/b_2 + (a_1-a_2)/b_2]}$$

$$\text{GS } C.\ acutatum = \text{Mass single genome} / 1.096\times10^{-21}$$

with 1.096×10^{-21} g being the average mass of a single base pair and $Ct = b_1x_1 + a_1$ and $Ct = b_2x_2 + a_2$ the linear regression curves obtained after simultaneous real-time PCR with genomic DNA (gDNA) and plasmid DNA of the single-copy β-tubulin 2 gene, resp.

The ITS copy number per genome was calculated by:

$$\#\text{ ITS copies} = \text{Mass single genome} \times 10^{[b_3X_3/b_4 + (a_3-a_4)/b_4] - X_3}$$

with $Ct= b_3x_3 + a_3$ and $Ct =b_4x_4 + a_4$ the linear regression curves obtained after simultaneous real-time PCR with gDNA and plasmid DNA of the multi-copy ITS region, resp.

Infected strawberry leaves

-Leaves were artificially inoculated. For the sensitivity tests, 100% infected leaf tissue was serially diluted with healthy leaf tissue and infected leaf samples were taken at various times after inoculation.
-Naturally infected leaves collected from production fields and planting material were analyzed using the developed real-time PCR.

Results

Development and sensitivity of the real-time PCR

Using TaqMan technology, the ITS-based assay could reliably detect and quantify as little as 50 fg of gDNA, 100 copies of cloned target DNA, or 25 conidia. The β-tubulin-based assay was circa 66 times less sensitive than the ITS-based assay (**Figure 2**), and therefore less suitable for detection purposes.

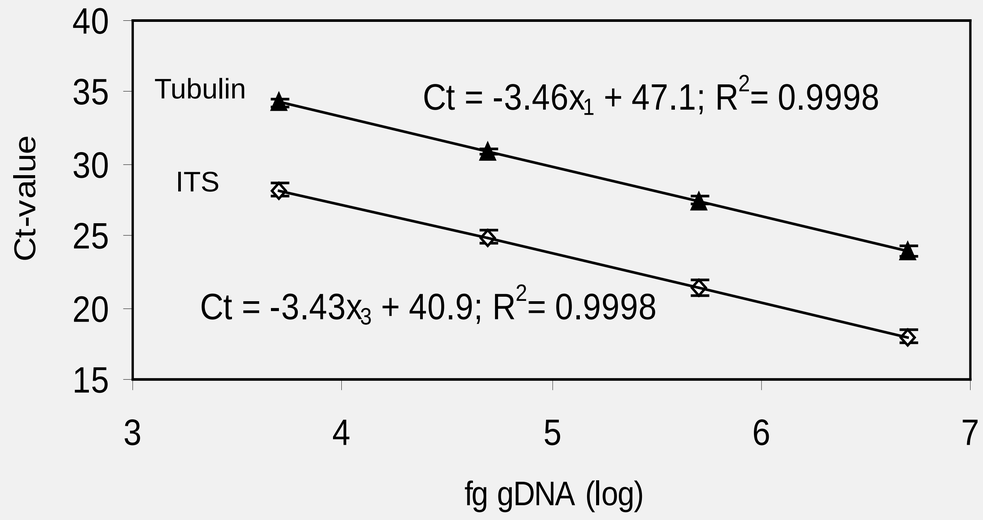

Figure 2. Standard curves from SYBRGreen real-time PCR using ITS- & β-tubulin-based primer sets and gDNA from *C. acutatum*

Genome size and ITS copy number of *C. acutatum*

We estimated a genome size of 60 Mbp and the presence of circa 20 copies of the ITS region in one genome of *C. acutatum*.

Detection of *C. acutatum* in inoculated strawberry leaves

We were able to detect *C. acutatum* in plant tissue mixes of which only 0.001% of the tissue was artificially infected by *C. acutatum*. In addition, the assay allowed monitoring of symptomless growth of *C. acutatum* on strawberry leaves and detection of *C. acutatum* in naturally infected but symptomless strawberry leaves.

Conclusion

The real-time PCR assay developed in this study
- is highly specific and sensitive for *C. acutatum*
- proved useful in revealing insights into the genome of *C. acutatum*
- can be used in routine quarantine inspections to screen strawberry plants
- can be used in the study of the biology of *C. acutatum* on strawberry plants

Reference

Lee C, Lee S, Shin SG, Hwang S (2008). Real-time PCR determination of rRNA gene copy number: absolute and relative quantification assays with *Escherichia coli*. *Applied Microbiology and Biotechnology* 78, 371-376.

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