Development of a Multiplex TaqMan Real-Time PCR Assay for Sensitive and Rapid Detection of Phytoplasmas Infecting *Rubus* Species

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Abstract

Rubus stunt, a disease associated with phytoplasma infections in wild and cultivated Rubus species, is a major challenge in the production of raspberries, blackberries, and loganberries. Phytoplasmas are cell wall-less bacteria inhabiting the phloem and are transferred by phloem feeding insects. As the time between infection of a plant and the development of disease symptoms can take up to 1 year, an early detection of phytoplasmas using highly sensitive and rapid molecular methods is of great importance to minimize their spread. Therefore, in this study, a multiplex real-time PCR assay using TaqMan probes in combination with four different kinds of fluorogenic dyes in the same reaction was developed, allowing a rapid and simultaneous detection of different groups of phytoplasmas infecting Rubus species. This assay is a solid tool for the screening of mother plants in nurseries and for further research in phytoplasmas infecting Rubus species.

Keywords: Phytoplasma; Rubus stunt; Real-time PCR; TaqMan; Diagnosis.

Introduction

Rubus stunt is an economically important disease in the production of raspberries (*Rubus ideaeus* L.), blackberries (*Rubus fruticosus* L.), and loganberries (*Rubus* x *loganobaccus*). It is caused by phytoplasmas, a group of cell wall-less plant pathogenic bacteria that are found in the phloem sieve tubes of plants (Lee *et al*., 2000) and in phloem sucking homopterous insects (Christensen *et al*., 2005). Infected plants may show one or several of the following symptoms: stunting, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation and fruit malformations (Mäurer & Seemüller, 1994). The time between infection of a plant and the development of disease symptoms can take up to 1 year. This especially poses a problem since nurseries produce *Rubus* plants by vegetative propagation. Therefore, in order to prevent the spread of Rubus stunt, it is of great importance to test mother plants prior to propagation. However, the currently available method to detect phytoplasmas in plant tissue, namely nested PCR (polymerase chain reaction) with phytoplasma specific primers, meets some difficulties: it is time consuming, has an increased chance of contamination compared to regular PCR and therefore a higher risk of false results. Thus far, phytoplasmas belonging to the 16Sr groups of elm yellows (16SrV), X disease (16SrIII), and aster yellows (16SrI) have been identified in *Rubus* species. In this study, a new multiplex real-time PCR assay using TaqMan probes was developed for the rapid and simultaneous detection of different groups of phytoplasmas infecting *Rubus* species.

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Material and Methods

Elm yellows group specific primers and TaqMan probes were designed for the *secY* and *degV* genes of *Candidatus* Phytoplasma rubi. The *secY* gene of *Ca*. Phytoplasma pruni was used to generate X disease specific primers and probes and the *tuf* gene of *Ca*. Phytoplasma asteris was used for the aster yellows group. All primers and probes were designed using IDT's PrimerQuest (https://eu.idtdna.com/PrimerQuest/Home/Index). For the universal detection of phytoplasmas a primer and probe pair from Christensen *et al*. (2004) was used. In addition, a primer and probe set developed by Oberhänsli *et al*. (2011) for 18S rDNA detection of plant DNA was used as an internal control to allow the detection of plant host DNA in the same reaction, enabling the conformation of a successful DNA extraction and to exclude false negatives due to potential inhibition of the PCR. TaqMan probes were labelled with a combination of 4 different fluorogenic dyes allowing simultaneous detection of up to 4 targets in a single reaction.

DNA was extracted from 1 g of leaf or root tissue of healthy and symptomatic *Rubus* species using an initial phytoplasma enrichment step with phosphate grinding buffer prior to the CTAB extraction according to Prince *et al*. (1993). Primers and probes were run in 20 µl reactions employing the iQ Multiplex Powermix (Biorad) on an iQ5 real-time thermal cycler (Biorad) with an initial denaturation step of 2 min at 95 °C followed by 45 cycles with 10 sec denaturation at 95 °C and 60 sec annealing and elongation at 55 °C.

Results and Discussion

A multiplex real-time PCR for detection of phytoplasmas infecting *Rubus* species was successfully developed. The internal control allowed detecting false negative results due to substances in the DNA extract inhibiting PCR or failed DNA extractions. It was possible to detect phytoplasmas in DNA extract dilutions from 1:5 to 1:1000 with Ct (Cycle threshold) values ranging between 29 and 38 (Fig. 1).

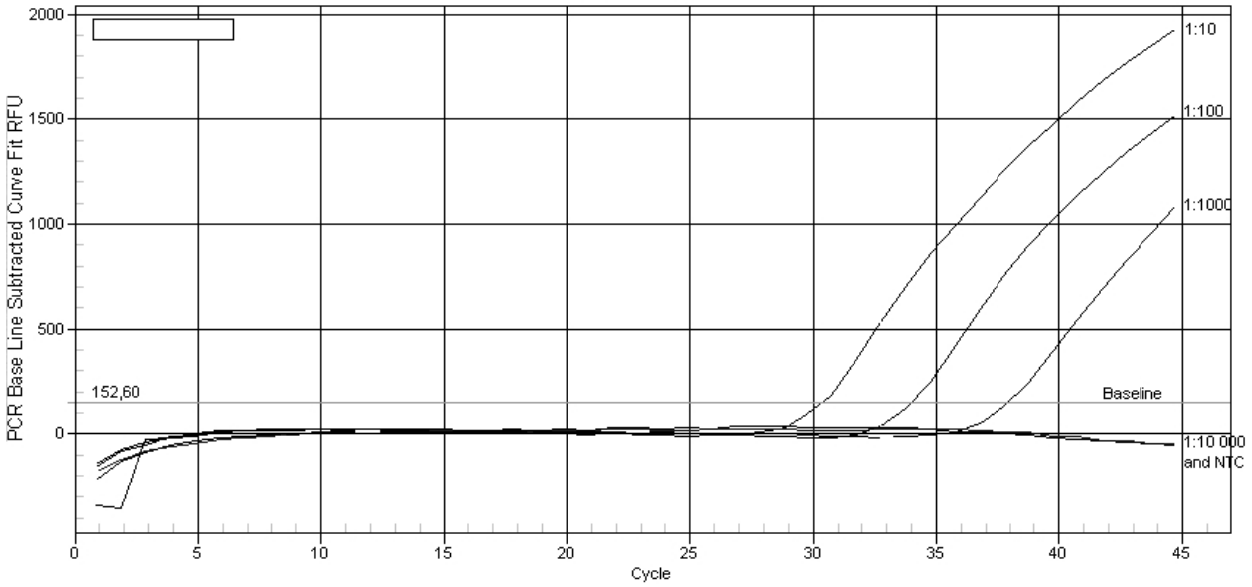

Figure 1: Amplification chart for the elm yellows group specific TaqMan probe generated from the *secY* gene of *Ca.* Phytoplasma rubi. The DNA extract of raspberry roots was assayed at 4 different dilutions, namely 1:10, 1:100, 1:1000, and 1:10 000 with an additional no template control (NTC). Ct values reflect the cycle number at which the fluorescence generated within a reaction crosses the baseline threshold. In this case, at Ct values of 30.38 for the 1:10, at 33.99 for the 1:100 and at 37.97 for the 1:1000 dilution of the samples. There was no positive amplification for the 1:10 000 dilution and the NTC.

TaqMan assays for detection of phytoplasmas were shown to be at least as sensitive as nested PCR (Smart *et al*., 1996; Angelini *et al*., 2007), but less susceptible to inhibitors. This leads to higher detection sensitivity of TaqMan assays due to the fact that DNA extracts could be used less diluted (Oberhänsli *et al*., 2011). Compared to nested PCR, the multiplex TaqMan assay developed in this study is time saving, has a reduced risk of contaminations and is therefore more reliable. These facts, in addition to the simultaneous detection of different groups of phytoplasmas and an internal control, are making the developed assay in this study a solid tool for the screening of mother plants in nurseries and for further research on phytoplasmas infecting *Rubus* species. The cost of the assay is approximately 1.70 € per sample (labor excluded). This assay will now be used to monitor presence and distribution of phytoplasmas in *Rubus* plants of different geographic origins, cultivars and cultivation systems as well as in putative insect vectors like leafhoppers.

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