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Development of specific detection primers for '*Candidatus* phytoplasma pyri'

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Abstract

Pear decline (PD) is an economically highly important disease in many pear growing regions. It is associated with a phytoplasma of the 16Sr group X: '*Candidatus* Phytoplasma pyri'. Despite its importance, reliable detection primers for direct identification of the pathogen by PCR are missing. Available PD-specific primers in the 16S-23S rDNA sequence detected only 80% of PD-infected field-collected samples from Southwest Germany. In order to select new primers sequence data of the 16S-23S rDNA region were obtained for two strains of '*Ca.* P. pyri'. PD-specific primers were selected *in silico* and their specificity was tested with reference strains of the 16Sr group X. They proved to be highly specific for '*Ca.* P. pyri'. The reliability of the primers for PD detection was evaluated with field samples of pear. The detection rate could be improved up to 90% with respect to phytoplasma detection in the same samples with highly sensitive universal ribosomal primers.

Key words: Pear decline, *Pyrus*, PCR detection, 16S rDNA, sequence analysis.

Introduction

Pear decline (PD) is one of the most important diseases of pear and induces a more or less quick decline of the tree. The disease presumably originates from Europe and has been introduced into North America as well as its vector (Seemüller, 1989). Today, PD probably occurs wherever pear is grown in Europe and North America. Recently, PD has also been reported from the Asian part of Turkey (Sertkaya *et al.,* 2008) and a similar decline of pear has been found in Taiwan (Liu *et al.,* 2007). New outbreaks of PD are currently observed across Europe.

Restriction fragment length polymorphisms (RFLP) and sequence analysis of ribosomal DNA (rDNA) revealed that the disease is associated with a distinct phytoplasma, '*Candidatus* Phytoplasma pyri', that is closely related to phytoplasmas associated with other important diseases of fruit crops: '*Candidatus* Phytoplasma mali' causing apple proliferation (AP) and '*Candidatus* Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY) (Seemüller and Schneider, 2004). They all belong to the 16Sr group X, the Apple proliferation cluster.

Three psyllid species of the genus *Cacopsylla* are recognised or presumed vectors of PD: *Cacopsylla pyri* (Linnaeus), *C. pyricola* (Foerster) and *C. pyrisuga* (Foerster) (reviewed by Jarausch and Jarausch, 2010). As PD is of high economic relevance reliable detection of the pathogen in plants and insects is of paramount importance for sanitary measures as well as for insect vector identification. Currently, PCR technology is the method of choice for sensitive detection of phytoplasmas in woody plants and insects. Up to now, universal phytoplasma primers and specific primers both derived from the 16S rDNA sequence and the intergenic 16S-23S rDNA region are most widely used. However, due to the close relationship of '*Ca.* P. pyri' with '*Ca.* P. mali' and '*Ca.* P. prunorum' most of the specific primers show cross reactivity with the DNA of other AP-group fruit tree phytoplasmas, especially with '*Ca.* P. mali'. Differentiation of the pathogens can only be achieved by RFLP analysis (Lorenz *et al*., 1995) and sequence analyses. So far, only one of the ribosomal primer pairs (fPD/rPDS) shows a higher specificity and does not cross-amplify the target from '*Ca.* P. mali'. However, not all strains of '*Ca.* P. pyri' can be detected with these primers (Lorenz *et al.*, 1995). Thus, the objective of this study was to improve the reliability of the PD detection primers to allow a one-step identification of the pathogen in plants and insects.

Materials and methods

Plant material was derived from symptomatic and non-symptomatic pear trees in the Southwest of Germany and was sampled in late autumn when the concentration of the phytoplasma in the tree is highest. Phloem was prepared from branches and was extracted with a CTAB-based protocol as described by Maixner *et al.* (1995). Phytoplasma reference strains were maintained in micropropagated pear ('*Ca.* P. pyri', strain PD9267) as described in Jarausch *et al.* (2000), in micropropagated apple ('*Ca.* P. mali') as published by Jarausch *et al.* (1996) or in micropropagated *Prunus* ('*Ca.* P. prunorum') (Jarausch *et al.*, 1994). Shoot material from these plants was extracted with the same protocol.

PCR amplification of phytoplasma DNA was achieved with universal ribosomal primers fU5/rU4 (Ahrens and Seemüller, 1992) or with 16Sr group X-specific primers fO1/rO1 (Lorenz *et al.*, 1995). In addition, PD-specific ribosomal primers fPD/rPDS were applied (Lorenz *et al.*, 1995).

For sequence analysis, PCR products were cloned and sequenced with standard procedures.

Results and discussion

In total, 122 samples of pear trees were proven to be infected by phytoplasmas using PCR detection with universal ribosomal primers fU5/rU4. Almost all samples yielded specific PCR products with primers fO1/rO1. However, '*Ca.* P. pyri' was detected in only 80% of the samples. This is in agreement with the data reported by Lorenz *et al.* (1995). This failure of detecting all '*Ca.* P. pyri' strains can be due to an insufficient sensitivity of the primers or to genetic differences of the phytoplasma strains. Therefore, sequence data were produced for two strains for the 16S rDNA and the 16S-23S rDNA intergenic region. One sequence was obtained from the reference strain PD9267, the other from a field-collected isolate from Southwest Germany. Both sequences were almost identical to all other complete 16S-23S rDNA sequences of '*Ca.* P. pyri' available in Genebank. One exception was the PD phytoplasma sequence accession Y16392 which has two important deletions compared to the other '*Ca.* P. pyri' sequences. The primer fPD as well as the primer rPDS were both selected in these regions and their *in silico* specificity with regard to other 16Sr group X phytoplasmas is mainly based on these deletions. Corrected primers proved to be no longer specific when tested with other European fruit tree phytoplasmas. Therefore, new primers were selected in the 16S-23S rDNA sequence region and their specificity for '*Ca.* P. pyri' was first checked *in silico*. The specificity of the primers was then evaluated with micropropagated plants highly infected with '*Ca.* P. mali' and '*Ca.* P. prunorum', respectively. No cross hybridization with these most closely related phytoplasmas was observed. The sensitivity and reliability of PD detection was then tested with the 122 field-collected pear samples tested positive with universal primers. Ninety percent of the samples yielded a specific PCR product with the new PD-specific primers. The majority of the non-reacting samples did not give rise to a PCR product with primers fO1/rO1 either, indicating a very low concentration of phytoplasma DNA. For these samples, the application of nested PCR approaches will be needed.

In conclusion, we developed specific primers for the direct detection of '*Ca.* P. pyri'. Further studies with PD samples from different geographic regions are needed to confirm that the primers detect all strains of '*Ca.* P. pyri'. The primers will be very valuable especially for the work with the different insect vectors of '*Ca.* P. pyri' which might be infected with different types of phytoplasmas in nature.

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