Establishment of a quantitative real-time PCR assay for the specific quantification of Ca. *Phytoplasma prunorum* in plants and insects

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Abstract

A real-time PCR assay for the quantification of Ca. *Phytoplasma prunorum* has been established which combines the specificity of detection with a low cost method of quantitative PCR. The assay uses the specific primers ECA1/ECA2 with a SYBR Green I protocol. A gene fragment of Ca. *P. prunorum* with the target of the primers has been cloned and is used as standard for quantification by the standard curve method. The assay has been successfully applied to measure the concentration of Ca. *P. prunorum* in insects as well as in different kinds of plant samples.

Keywords: European stone fruit yellows, *Cacopsylla pruni*, resistance screening

Introduction

European stone fruit yellows (ESFY) is an economically important decline disease of stone fruits (*Prunus* spp.) in Europe causing the highest losses in apricot, Japanese plum and peach (Lorenz et al., 1994). This quarantine disease is associated with the phloem-limited Candidatus *Phytoplasma prunorum* which is transmitted in nature by the psyllid *Cacopsylla pruni* (Carraro et al., 1998; Jarausch et al., 2001) and by man through dissemination of latently infected planting material or grafting. Ca. *P. prunorum* is closely related to other European fruit tree phytoplasmas like Ca. *Phytoplasma mali*, the agent of apple proliferation disease, and Ca. *Phytoplasma pyri*, the agent of pear decline (Seemüller and Schneider, 2004). Phytoplasma detection is routinely achieved by PCR and primers specific for Ca. *P. prunorum* are available (Jarausch et al., 1998). In recent years, determination of phytoplasma concentration has gained increasing importance for the analysis of the disease spread by the insect vector (Thébaud et al., 2009a) or for the evaluation of resistance *in vivo* (Bisognin et al., 2008a) as well as *in vitro* (Bisognin et al., 2008b). Quantification of the phytoplasma was achieved in these studies by applying real-time PCR and different assays have been published for the quantification of Ca. *P. mali* (Baric and Dalla Via, 2004; Jarausch et al., 2004), Ca. *P. prunorum* (Martini et al., 2007; Thébaud et al., 2009b) or both (Torres et al., 2005).

The objective of this study was to establish a low cost method for the routine quantification of Ca. *P. prunorum* in insects as well as in different kinds of plant samples based on the well established detection primers ECA1/ECA2 (Jarausch et al., 1998).

Material and methods

Total DNA from plant and insect samples was extracted with a modified CTAB-based protocol as described by Maixner et al. (1995). Phloem-preparations were used from field samples for extraction. PCR with primers ECA1/ECA2 was done as described (Jarausch et al., 1998) using *Taq* polymerase furnished by 5 Prime (Germany). PCR product cloning and subsequent sequencing were done according to standard procedures. Real-time PCR was carried out in a MJ Research Chromo4 cycler using white plates (ABgene, UK) and the standard PCR reaction mix supplemented with SYBR Green™ I (1:66000 dilution; AMRESCO, USA) with the Mg2+ concentration adjusted to 3 mM. Primer concentrations were 0.1 µM for primer ECA1 and 1 µM for primer ECA2. The quantification was done by using the standard curve quantification method with a serial dilution of a plasmid preparation containing the ECA1/ECA2 gene fragment. For phytoplasma quantification in insects the plasmid was diluted in total DNA extract of healthy *C. pruni*, to quantify the phytoplasma in plant samples the plasmid was diluted in total DNA extracts obtained from healthy *Prunus* maintained *in vivo* or *in vitro*, respectively. Cycle conditions were for 40 times 15 s at 95°C, 30 s at 54°C, and 30 s at 69°C followed by a plate read. The qPCR was completed by a melting curve analysis.

Results and discussion

A Ca. *Phytoplasma prunorum*-specific PCR product was amplified with primers ECA1/ECA2 from a naturally ESFY-infected apricot from Southwest Germany, cloned and sequenced. The obtained plasmid preparation was used as a standard in a 10-fold dilution series ranging from 10^8 to 10^1 to establish the quantitative PCR (qPCR) assay. A SYBR Green™ I protocol was used as previously published (Jarausch et al., 2004) with one major modification: the use of a commercial qPCR kit was replaced by adding SYBR Green™ I to a normal PCR reaction. Mg2+ concentration was

adjusted to 3mM and the primer concentrations had to be altered to avoid primer dimer formation. The analysis of the standard curve showed that an efficient amplification could be achieved with this protocol (Fig. 1). The qPCR assay was first tested with insect samples and Ca. *P. prunorum* was reliably quantified in samples from field collected individuals of *C. pruni* (Fig. 2). The phytoplasma concentration measured in overwintered re-migrant adults of the vector *Cacopsylla pruni* was in the range of published data (Thébaud et al., 2009a). The test was also successfully applied to quantify the phytoplasma in different micropropagated *Prunus* plants after graft-inoculation *in vitro* as described previously (Jarausch et al., 1999). The assay worked equally well for the quantification of Ca. *P. prunorum* in plant samples obtained from the field. In both kinds of plant samples the phytoplasma concentration was correlated to the fresh weight of plant material used for DNA extraction. In both kinds of tissue comparable phytoplasma concentrations were measured in the range of 10^9 phytoplasma genome copies per g freshweight.

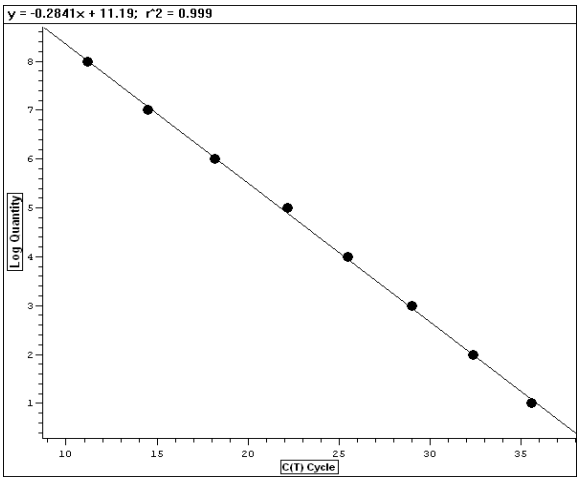

Fig. 1 Standard curve of a serial dilution 10^8 to 10^1 of a plasmid containing the ECA1/ECA2 PCR fragment and amplified in the established qPCR assay; Ct values are plotted against the log plasmid copies.

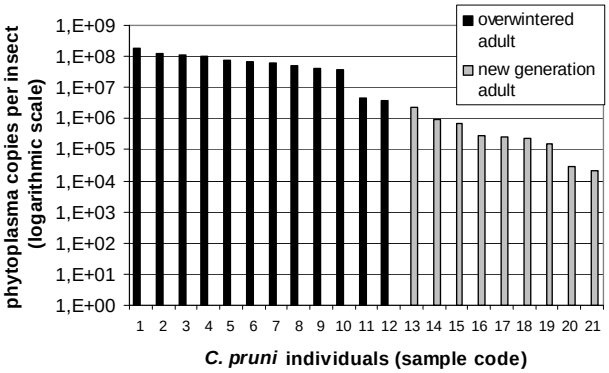

Fig. 2 Quantification of Ca. *P. prunorum* in single adults of *Cacopsylla pruni* at different time points of their life cycle expressed as phytoplasma genome copies per insect.

The established qPCR assay offers the advantage of the use of well established detection primers in combination with a low cost protocol based on SYBR Green™ I technology for the specific quantification of Ca. *Phytoplasma prunorum* in plants and insects. The assay could already be in routinely applied for the determination of the phytoplasma load of

naturally infected vector individuals of *Cacopsylla pruni* as well as for the evaluation of the phytoplasma concentration in inoculated genotypes of *Prunus* species *in vitro* and *in vivo*.

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