Comparison of European stone fruit yellows phytoplasma strains differing in virulence by multi-gene sequence analyses

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Abstract

Twenty strains of the ESFY phytoplasma, which on the basis of graft-inoculation experiments greatly differ in aggressiveness, were examined by sequence analyses of several PCR-amplified non-ribosomal genes in order to identify molecular markers linked to virulence. These strains, which were maintained in *P. insititia* rootstock St. Julien GF 655/2 were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and RFLP analyses of PCR-amplified rDNA. Also, the virulent ESFY strains maintained in periwinkle, namely GSFY1, GSFY2 and ESFY1, as well as an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in recovered apricot trees in France and used there as a cross protecting agent, were included in the work for comparison. For PCR amplification, primers were designed from a number of genes distributed over the chromosome of the closely related apple proliferation phytoplasma strain AT. Visible PCR products were only obtained with primer pairs derived from the *tuf* gene which encodes the elongation factor Tu (EF-Tu), *rps*C (*rps*3) gene encoding the ribosomal protein S3, *tly*C gene which encodes a hemolysin known as a membrane-damaging agent and important virulence factor of many bacteria, the *imp* and *fol* genes encoding an immunodominant membrane protein and an enzyme involved in the folate biosynthesis, respectively. Nucleotide sequence comparisons revealed that the highest genomic variability occurred within the *imp* gene sequence with dissimilarity values ranging from 0.2 to 4.6%. For the remaining genes, the strains examined proved to be identical or nearly identical. Within the *tuf* gene, an extra *Taq*I site known to occur in strain GSFY1 was not identified in other strains. The genetic differences observed among the strains examined are neither suitable markers for strain differentiation nor linked to pathological traits.

Keywords: European stone fruit yellows, strain virulence, 16SrX group, *tly*C gene, *Prunus* spp.

Introduction

The European stone fruit yellows (ESFY) agent 'Candidatus *Phytoplasma prunorum*' is an important prokaryotic pathogen that infects most or all kinds of stone fruits in Europe and is known to cause apricot chlorotic leaf roll of apricot (*Prunus armeniaca*), leptonecrosis and decline of Japanese plum (*P. salicina*), yellows and decline diseases of peach (*P. persica*), Molières disease of sweet cherry (*P. avium*) and European plum (*P. domestica*), and other diseases that include those affecting almond (*P. dulcis*) and flowering cherry (*P. serrulata*) (Lorenz et al., 1994). This organism is closely related to important fruit trees pathogens like apple proliferation (AP), pear decline (PD) and peach yellow leaf roll (PYLR) agents. Together they form a distinct phylogenetic cluster, the AP- or 16SrX group (Seemüller et al., 1998; IRPCM, 2004). Previous work has shown that strains of the ESFY phytoplasma greatly differed in virulence when examined by graft inoculation of trees on peach, peach hybrid GF 677 and *Prunus* 'Marianna' GF 8/1 rootstocks. While some strains were nearly avirulent or weakly virulent and induced only mild foliar symptoms and slightly reduced vigor but no mortality, others were highly virulent and caused severe symptoms and a high mortality rate of affected trees (Kison and Seemüller, 2001). However, the strains showing pathological differences were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) as well as Southern blot hybridization using probes suitable for the differentiation of the fruit tree phytoplasmas of the AP group (Kison and Seemüller, 2001; Seemüller and Schneider, 2004). Knowledge of genes mediating pathogenicity and virulence is urgently needed for insights into phytoplasma pathology and for studying plant resistance against phytoplasmas. Thus, twenty differently virulent strains of the ESFY phytoplasma were examined by analysing the sequence of several PCR-amplified non-ribosomal genes in order to attempt to identifymolecular markers linked to virulence.

Materials and methods

Phytoplasma Sources. Twenty strains of the ESFY phytoplasma differing in virulence that were maintained in *P. insititia* rootstock St. Julien GF 655/2 in the experimental field at institute in Dossenheim, were examined (Table 1). Also, the virulent ESFY phytoplasma strains maintained in *Catharanthus roseus* (periwinkle), namely GSFY1, GSFY2 and ESFY1 (Marcone and Seemüller, 2001) as well as four isolates (F1, F2, F3 and F4) of an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in a recovered apricot tree in France and used there as a cross protecting agent to control the apricot chlorotic leaf roll disease (Morvan et al., 1986; Castelain et al., 1997), were included in the work for comparison. The latter isolates are also indistinguishable from severe strains of ESFY phytoplasma on the basis of RFLP analysis of PCR-amplified rDNA (unpublished data). DNA samples from strain AT of the AP phytoplasma were also included in this study. DNA Isolation. From trees either petioles, midribs, or phloem tissue from stem portions and roots, approximately 3.0 cm in diameter, were used. Phloem tissue was prepared as described (Ahrens and Seemüller, 1994). Young shoots including leaves were taken from periwinkle. DNA was isolated from approximately 1.0 g of fresh tissue using a phytoplasma-enrichment procedure as described previously (Ahrens and Seemüller, 1992).

Tab. 1 German strains of the ESFY phytoplasma maintained in stone fruit genotypes in the field and examined in this study.

Strain designation	Designation[a]	Virulence	Vigor reduction
G1*		+	Moderate
G2	Peach 4	(+)	No
G3	Peach 2	(+)	No
G4	Peach 3	+	Moderate
G5	Almond 6	(+)	No
G6	Almond 1	++	Severe
G7	Almond 7	+	Moderate
G8	Almond 4	+	Moderate
G9	Almond 3	++	Severe
G10	Almond 8	(+)	No
G11*		+	Moderate
G12	Apricot 2	(+)	No
G13	Apricot 1	++	Severe
G14*		(+)	No
G15	Apricot 3	(+)	No
G16	Apricot 4	(+)	No
G17	Japanese plum	++	Severe
Gapr1*		++	Severe
Gapr2*		++	Severe
Gapr3*		+	Moderate

[a] Strain designation given by Kison and Seemüller (2001); * Not clearly described.

Primers and PCR amplification. For PCR amplification of ESFY fragments, primers were derived from 'Candidatus *Phytoplasma mali*' strain AT genes listed in Table 2 for which the complete sequence is available (Kube et al., 2008). Amplification was performed in 25-µl reactions containing 0.5 µM of each primer, 0.1 mM each dNTPs, 0.6 U of heat-stable polymerase (Invitrogen), and 1x polymerase buffer. The reaction was subjected to 35 cycles in a thermal cycler (Stratagene) at the following parameters: 95 ºC for 1 min, 50 ºC (48 °C for primers fFol/rFol, and 52 °C for primers fTlyC/rTlyC) for 1 min, and 70ºC for 1 min (10 min for the final cycle). Five microliters of PCR product were analyzed by electrophoresis in a 1.5 % horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in the presence of 0.5 µg/ml ethidium bromide. DNA bands were visualized using a UV transilluminator.

Tab. 2 Oligonucleotide primers used in this study.

Primer	Primer sequence (5'-3')	Sense	Target (gene)
fTuf	GCA AAT GGA CGC TGG TAT TT	Forward	Tuf
rTuf	ACA TTA TAG AAT GGT TAA ATA AGC	Reverse	Tuf
fRpsC	TTG GGA TTC TAA ATG GTT TGC	Forward	RpsC
rRpsC	TTT CGC CTG GTA AAA CAT CA	Reverse	RpsC
fTlyC	CGG TTT AAT GGT TCC TTT CG	Forward	TlyC
rTlyC	CCT GAT AAA ACA AAT AAA TGC CAA A	Reverse	TlyC
fImp	CAA ATG ATA AAG CTG ATC AA	Forward	Imp
rImp	CAC ATC CTT TGT TTA AAA ATT TTA T	Reverse	Imp
fFol	TAA TAT GCT TCC TTG GCA TT	Forward	Fol
rFol	CAA CAA AAA TTA ATT CGG GAT A	Reverse	Fol

Sequence analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and then sequenced either directly or cloned prior to sequencing. For cloning, DNA fragments were ligated into plasmid vector pGEM-T (Promega) and recombinant plasmids used to transform *Escherichia coli* strain DH5α. Sequencing of both strands was performed using a dideoxy chain termination chemistry. Primers for direct sequencing of PCR products were the same as for PCR amplification whereas the standard primers pUC/M13 forward and pUC/M13 reverse were used for sequencing the cloned fragments. Sequence alignments were performed by using CLUSTAL, version 5, with LaserGene software (DNASTAR).

Results and discussion

With PCR assays, visible PCR products from all strains tested were only obtained with the primer pairs fTuf/rTuf, fRpsC/rRpsC, fTlyC/rTlyC, fImp/rImp and fFol/rFol. Primers fTuf/rTuf derived from the *tuf* gene encodes the elongation factor Tu (EF-Tu) and mediates the transport of aminoacyl-tRNA to the codon recognition site of ribosomes; primers fRpsC/rRpsC derived from the *rps*C (*rps*3) gene encoding the ribosomal protein S3 is an essential component of ribosomes; fTlyC/rTlyC were from *tly*C gene which encodes a hemolysin, a membrane-damaging agent which has been implicated as a virulence factor for a variety of human pathogens, mainly Gram-positive bacteria (Radulovic et al., 1999); and, fImp/rImp and fFol/rFol from the *imp* and *fol* genes encoding an immunodominant membrane protein and an enzyme essential for folate biosynthesis, respectively. The sizes of the amplified PCR fragments were identical to those obtained from strain AT. Other selected primers failed to amplify the target DNA from ESFY phytoplasma strains at different annealing temperatures. A representative number of amplimers from strains of each virulence category was sequenced directly or after cloning.

Nucleotide sequence comparisons revealed that the highest genomic variability occurred in the *imp* gene with similarity values ranging from 95.4 (GSFY1 *versus* G17) to 99.8 % (F1 *versus* G17). The highest sequence similarity value was shared by two differently virulent strains, the avirulent strain F1 and the severe strain G17, whereas the greatest dissimilarity occurred between two strains of the same virulence category, namely GSFY1 and G17, both virulent. For *tuf* and *tly*C genes, the strains examined proved to be identical or nearly identical with similarity values between 99.7 and 99.9 %, and 99.8 and 100 %, respectively. No dissimilarities were observed in both *rps*C and *fol* genes. Within the *imp* gene, the polymorphisms observed in the sequence of a given strain were usually not shared by the strains of the same virulence category. For instance, the polymorphism at position 641 where T was replaced by a C residue, in the mild strain G3, did not occur in other mild or avirulent strains such as F1 and G1. Similar results were obtained for *tly*C and *tuf* genes. In the *tly*C gene, the polymorphism at position 50 where G was replaced by an A residue occurred in both virulent and avirulent strains. In the *tuf* gene, the substitution of C with an A residue in the virulent strain GSFY2 at position 840, did not occur in the other virulent strains GSFY1, G17, ESFY1 and G13. Also, within the *tuf* gene, the presence of an additional *Taq*I restriction site, in the virulent strain GSFY1 following position 429, due to a substitution of T with a C residue, was not identified for the other strains. This additional *Taq*I restriction site, which had already known to occur in the strain GSFY1, is responsible for differences in restriction profiles as confirmed by RFLP analysis of PCR-amplified *tuf* gene sequences (Marcone et al., 2002).

Results of the present study confirm previous findings that ESFY phytoplasma strains showing pathological differences are relatively homogenous at the level of the molecular markers so far examined. Also, the genetic differences observed among the strains examined, mainly those occurring in the *imp* and *tuf* genes are neither suitable markers for strain differentiation nor linked to pathological traits. Thus, further comparisons of DNA fragments from avirulent and virulent strains are needed to identify genes mediating pathogenicity or virulence. The most promising approach would be the sequence comparison of the entire chromosome from an avirulent and severe strain.

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