

Molecular differentiation of severe and mild strains of ‘*Candidatus Phytoplasma mali*’ and evidence that their interaction in multiply infected trees determines disease severity

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Abstract

Previous work has shown that multiple infections of apple trees by distinctly different strains of ‘*Candidatus Phytoplasma mali*’ are widespread. In the current study, pathological data of infected trees with single or multiple phytoplasma were analyzed and compared with molecular data based on a *hflB* gene of the infecting phytoplasmas. Single-strand conformation polymorphism and sequence analysis of a variable *hflB* gene fragment revealed that mild and severe strains can be distinguished by their SSCP profiles and their phylogenetic clustering. Analysis of cloned sequences from mild and severe single-strain accessions resulted in two groups of reads that clustered, according to their virulence, distantly in the phylogram. Based on this data, the clustering patterns of multiple-strain accession sequences indicated that nearly all of them were composed of mild and severe strains. Our data indicate that the virulence of multiple-strain accessions is determined by the ratio of the occurring mild and severe strains in that mild accessions were characterized by the predominance of sequences representing mild strains and vice versa. There is evidence that shifts in the population may occur that drastically alter virulence of multiple-strain accessions.

Key words: Apple proliferation, ‘*Candidatus Phytoplasma mali*’, virulence, multiple infections, classification.

Introduction

Apple proliferation (AP), associated with ‘*Candidatus Phytoplasma mali*’, is one of the most important phytoplasma diseases in Europe. AP induces a range of symptoms that are either specific, such as witches brooms, rosettes, and enlarged stipules, or largely nonspecific, such as foliar reddening, yellowing, growth suppression and undersized fruits. However, symptom expression is often subject to fluctuation. Diseased trees may recover and may show no or only mild symptoms for shorter or longer periods after which severe symptoms may reappear. Symptom development also depends on the virulence of the infecting AP phytoplasma strains (Seemüller and Schneider, 2007).

‘*Ca. P. mali*’ is, at the level of ribosomal DNA sequences, a homogenous species throughout Europe. To elucidate the molecular diversity within this taxon, several other approaches have been employed. However, the genotypes delineated in these efforts were unrelated to phenotypical traits such as virulence and other pathological characteristics. High resolution of ‘*Ca. P. mali*’ genotypes was achieved by single-strand conformation polymorphism (SSCP) analysis of a fragment of the ATP00464-type *hflB* gene of strain AT (Schneider and Seemüller, 2009). In continuing this work, we identified DNA samples yielding more complex SSCP profiles that suggested the presence of multiple infections in a single tree. Cloning of PCR products of such samples resulted in clone populations showing distinct profile polymorphisms and diverse sequences that clustered distantly when subjected to phylogenetic analysis. The collective data indicated that

they were composed of two or three distinct ‘*Ca. P. mali*’ strains (Seemüller *et al.*, 2010).

Recent work also suggested that multiple infections are of pathological relevance, probably due to antagonistic strain interactions leading to shifts in the populations (Seemüller *et al.*, 2010). In the research presented here we characterized the AP phytoplasmas present in singly and multiply infected trees. By linking virulence of the infecting phytoplasmas with their SSCP and sequence data, it was possible to distinguish and classify mild and severe strains at the molecular level.

Materials and methods

Root or shoot scions from 27 donor trees naturally infected by ‘*Ca. P. mali*’ were collected in Germany, France and Italy to graft-inoculate healthy rootstocks. The recipient trees were grown in the open or in an unheated greenhouse. The occurrence of AP symptoms was annually recorded using a rating scheme from 0 (no symptom) to 3 (severe symptoms such as witches brooms, stunting or reduced fruit size). The figures obtained were used for evaluating both symptomatology and virulence.

Root and/or the current season’s shoot samples were collected from infected apple trees. DNA was extracted from phloem preparations of roots or shoots using a cetyltrimethylammonium bromide procedure (Seemüller and Schneider, 2007). PCR amplification was performed employing primer pair *hflB3_1/rHflB3* (5’-TTCTAGCTATTTCATCGTGAA-3’/5’-CGGCGGAT TAGTAGCTCC-3’) that specifically amplifies a 528-bp

fragment of ATP00464 *hflB* gene homologues from all AP phytoplasma strains (Schneider and Seemüller, 2009).

hflB gene fragments obtained by PCR amplification were ligated into pGEM-T Easy vector system (Promega) and transformed to *Escherichia coli* XL1 Blue cells (Stratagene). Colony PCR was employed to amplify the inserts of recombinant plasmids using the primer pair described above. The PCR products were subjected to SSCP analysis. Selected cloned fragments were sequenced using M13 forward and reverse primers. Alignment of sequences was performed with ClustalX2 (Thompson *et al.*, 1997). For phylogenetic comparison a 454-bp fragment was selected and analyzed employing the MEGA4 program using Neighbor-Joining parameters and bootstrap analysis (Tamura *et al.*, 2007).

Results and discussion

In the current study the virulence of 27 '*Ca. P. mali*' accessions collected from three different groups of trees was observed over six to 17 years. These trees showed all major traits of AP disease, namely expression of severe symptoms at the onset of disease, inconsistent symptomatology, recovery, reappearance of disease and great differences in the virulence of the infecting phytoplasmas. From our work, there is evidence that a major reason for the unusual symptomatology of AP is the obviously widespread occurrence of multiple infections. Our study revealed that about half of the accessions examined were multiply infected and were composed of two to five distinct strains. The real percentage of multiple infections is probably even higher considering the fact that sometimes many cloned fragments had to be examined to identify this phenomenon.

In our attempt to molecularly characterize the virulence of '*Ca. P. mali*' strains we investigated the relationship between symptomatology and molecular traits of the infecting phytoplasmas. This effort was greatly facilitated by the availability of single-strain accessions. Cloning of *hflB* gene fragments of severe and mild strains of such accessions resulted in two types of sequences that clustered separately in phylogenetic analysis. This fact and the sequence diversity among the various single-strain accessions enabled us to prove that nearly all multiple-strain accessions were composed of mild and severe strains and to classify the cloned sequences accordingly. The separated clustering of clones representing severe and mild strains was supported by a range of molecular markers at the nucleotide and amino acid level.

The molecular classification of the components of multiple-strain accessions reflects their virulence. Cloned sequences of highly virulent accessions clustered predominantly with sequences of severe strains whereas sequences of mild accessions clustered mainly with sequences of mild strains. However, shifts in the phytoplasma composition may occur that drastically alter virulence. There is indication that growing condi-

tions trigger such shifts that seem to play an important role in the symptomatology of AP phytoplasma-infected trees. Our data also indicate that another mechanism may exist leading to low virulence. A few accessions proved to be highly virulent in the field but did not develop any symptom following graft transmission and greenhouse growing. As sequences representing mild and severe strains were present in equal numbers, it is conceivable that virulence factors were inhibited by action of the mild strain components.

In this study, we succeeded to classify phytoplasma virulence at the molecular level. In this way, it was possible to resolve the phytopathogenic composition of multiple-strain accessions and to estimate their importance for symptom expression and course of disease. As shown for one accession, the potential of multiple-strain accessions is not restricted to symptom expression in apple alone; it also affects the host range. Following dodder transmission, two mild strains of this accession grew specifically in either periwinkle or tobacco. The early observations with aster yellows phytoplasmas (Freitag, 1964; Kunkel, 1955) indicate that multiple phytoplasma infections and strain interactions are not restricted to the AP agent but are probably widespread phenomena in phytoplasma. They are not only important to better understand symptomatology and course of disease, but they also affect detection (primer and antibody specificity), identification and classification.

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