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Development of specific *secA*-based diagnostics for the 16SrXI and 16SrXIV phytoplasmas of the Gramineae

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Abstract

Phytoplasmas are responsible for a range of diseases in the Gramineae in Sub-Saharan Africa and Asia including sugarcane grassy shoot (white leaf), rice yellow dwarf, napier grass stunt and Bermuda grass white leaf. In previous work, we have designed universal nested PCR primers based on the *secA* gene for amplification of DNA from phytoplasmas in most 16Sr groups. However, these primers did not appear to work well on phytoplasmas in the 16SrXI and XIV groups. In this work, we have designed specific sets of primers based on the *secA* gene that can be used in a nested PCR assay to amplify either the napier grass stunt phytoplasma or the sugarcane phytoplasma or the rice/bermudagrass phytoplasmas. These assays and *secA* sequences have also been used to redefine the taxonomic relationships between these phytoplasmas.

Key words: diagnostics, Gramineae, phylogenetics, phytoplasmas, *secA* gene.

Introduction

Phytoplasmas are responsible for many serious plant diseases in Sub-Saharan Africa and South Asia including in major cereals, fodder crops and weeds (Arocha and Jones, 2010). In maize (Americas and Europe), wheat (China), barley and oats (Europe), phytoplasmas of the 16SrI aster yellows type are associated with diseases such as maize bushy stunt and wheat blue dwarf, and these can be found in many plant species, not just the Gramineae. By contrast, the phytoplasmas of the Gramineae in Africa and Asia appear to be specifically adapted to the grasses. These include the 16SrXI sugar cane grassy shoot / white leaf diseases and the rice yellow dwarf disease (Arocha and Jones, 2010). In Africa, there is the 16SrXI napier grass stunt phytoplasma which was first recorded on napier grass (*Pennisetum purpureum*) only 11 years ago and for which the vector has been identified as *Maiestas banda* (Obura *et al.,* 2009). In addition, there is the closely related 16SrXIV bermuda grass white leaf phytoplasma of *Cynodon dactylon* and other grasses, that is widespread throughout Africa and Asia, vectored by *Exitianus capicola*.

To improve detection and taxonomic classifications of phytoplasmas, we have designed primers for PCR based on the *secA* gene (Hodgetts *et al.,* 2008; Bekele *et al.,* 2011), along with assays based on the plant *cox* gene to confirm that DNA extracts support PCR/LAMP amplification. In this work we describe the development of specific assays for the phytoplasmas of the Gramineae.

Materials and methods

Samples were obtained from 3 napier grass stunt infected plants (originally from Kenya but maintained at the University of Nottingham), 20 sugarcane plants exhibiting grassy shoot (10) and white leaf (10) symptoms from Sri Lanka, 1 rice plant exhibiting yellow dwarf symptoms from Sri Lanka, 3 bermuda grass plants exhibiting white leaf symptoms from Sri Lanka, 8 sugarcane samples exhibiting grassy shoot symptoms and 1 rice yellow dwarf sample from Nghe An Province, central Viet Nam and 1 bermudagrass, 1 setaria grass and 5 digitaria grass samples exhibiting yellowing/white leaf symptoms from Ethiopia. DNA was extracted using the CTAB DNA preparation method (Doyle and Doyle, 1990), and the presence of PCR amplifiable DNA confirmed using primers based on the *cox* gene (Tomlinson *et al.,* 2010). The presence of phytoplasma in these samples was confirmed using 16S rRNA universal primers, and samples were then used to develop and validate the secA primers as outlined in the results.

Results

Initial tests using the *secA* nested primers as described in Bekele *et al.* (2011) failed to amplify from the grass samples even though these had proven to be positive for the presence of phytoplasma using 16S rRNA primers. However, by reducing the annealing temperature to 45°C, PCR products were obtained using certain combinations of the different nested primers. These PCR products were subsequently sequenced and used to design more specific primers that could then be used to amplify reliably from all the samples at 53°C annealing temperature. These new specific primers are listed in table 1, defined by the organism they are specific for.

Following amplification with these primers, a phylogenetic analysis was conducted on the *secA* sequences obtained (figure 1). This analysis indicated that all the

Table 1. Sequences of the *secA* gene primers developed and used in this and previous studies.

Primer name	Sequence 5'-3'	Reference
SecAF1	GARATGAAAACTGGRGAAGG	Hodgetts *et al.* (2008)
SecAR3	GTTTTRGCAGTTCCTGTCATNCC	Hodgetts *et al.* (2008)
SecAF5	ASTCGTGAAGCTGAAGG + AGCTAAAAGAGA-ATTTGAAGG	Bekele *et al.* (2011)
SecAR2	CCNTCRCTAAATTGNCGTCC	Bekele *et al.* (2011)
NGSsecfor1	TATACWACWAATAGTGAAATWGG	Abeysinghe *et al.* submitted
NGSsecrev1	GATAAGTAATAGTAGCAGCAATTTCAG	Abeysinghe *et al.* submitted
NGSsecfor2	CGATGAAGTWGATTCTGTC	Abeysinghe *et al.* submitted
NGSsecrev2	AGCTTCTAAAGCTTGATGTAATCC	Abeysinghe *et al.* submitted
Sugarsecfor2	GATTCTGTCTTAATAGACGAAGCTAG	Abeysinghe *et al.* submitted
Sugarsecrev2	GTAAATTGATCTATTATCAAAACATTATTT	Abeysinghe *et al.* submitted
Ricesecfor2	GATTCTGTCTTGATAGATGAAGCAAG	Abeysinghe *et al.* submitted
Rice secrev2	GTAAATTGGTCTATAATTAAAATCTGATTA	Abeysinghe *et al.* submitted

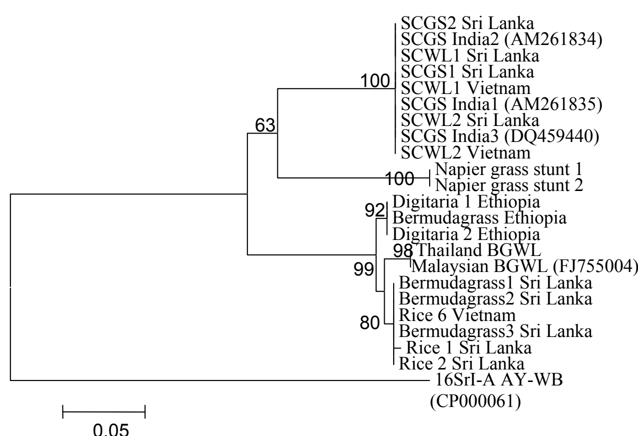

Figure 1. Phylogenetic tree based on the *secA* sequences obtained in this study and some obtained from GenBank (for which accession numbers are shown in parenthesis).

sugarcane phytoplasmas from Viet Nam and Sri Lanka were identical, and the same as the sequences that had been deposited in GenBank from India. These sequences were distinct from the Napier grass stunt *secA* sequences. There was then a separate cluster that contained the rice, bermudagrass and other grass sequences. Interestingly, these sequences appeared to be separated from each other on the basis of Country of origin rather than plant host, with the Ethiopian strains being slightly different from the Thailand/Malaysian strains which were in turn different from the India/Sri Lanka/Viet Nam strains.

Discussion

Primers have been designed in this study that can be used to differentiate between the phytoplasmas in the Gramineae in Africa and Asia based on the *secA* gene. This gene is more powerful than the 16S rDNA since it gives better resolution between the different groups, and is more reliable than use of the 16S-23S rRNA region, since there is only one copy of the *secA* gene, so less likely to be any problems due to heterogeneity between copies. The results based on the *secA* gene clearly differentiate the sugarcane, napier grass and rice/bermuda grass clusters from each other and also provide evidence that the rice/bermudagrass cluster can be separated on the basis of regional origin.

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