Molecular Diagnosis of Phytoplasmas

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Abstract

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Phytoplasmas are non-culturable, wall-less and phloem-restricted pathogens transmitted in a persistent manner by leafhoppers and planthoppers (Homoptera: Auchenorrhyncha) and psyllids (Homoptera: Sternorrhyncha). They are associated with diseases in many wild and cultivated plant species belonging to different families and cause economically important epidemics world-wide. The colonization of the plant by the phytoplasmas depends on the season, organ, host and pathogen species, and results in different symptoms due to complex interference with the host physiology. Sensitive and accurate diagnosis of these pathogens is crucial for the management of phytoplasma-associated diseases. Phytoplasmas are difficult to detect due to their low concentration especially in woody hosts and their erratic distribution in the infected plants. Their detection is now routinely done by nucleic acid-based techniques, mainly PCR. Total DNA preparations of good quality and enriched in phytoplasma DNA are usually obtained by including a time-consuming phytoplasma enrichment step, although simpler protocols have been developed using commercially available microspin columns. Successful phytoplasma detection in insect vectors may be attained with quicker total DNA extraction procedures, probably due to the high titre of the bacteria in the insect body. Universal phytoplasma-specific PCR primers have been identified in different positions of the ribosomal RNA operon, and group-specific primers have also been designed following comparison of the phytoplasma-specific 16SrRNA and 16S-23S intergenic regions of phytoplasmas belonging to different strain clusters. Ribosomal sequence-based primers are the most used for routine diagnosis of phytoplasmas. Universal and group-specific primers have also been targeted to other gene sequences, to sequences with no obvious predicted function and to the sequence of plasmids hosted by phytoplasmas. Routine diagnostic protocols usually involve the use of nested PCR. More recently phytoplasma diagnostic assays based on RT-PCR, real time PCR, PCR-ELISA, PCR-dot blot, heteroduplex mobility assay, 16S-23S spacer length polymorphism, microarray and nanobiotransducer hybridization have also been proposed.

Key words: Detection, Identification, RT-PCR, Real Time PCR, PCR-ELISA, PCR-Dot Blot.

Introduction

Phytoplasmas are non-culturable, wall-less and phloem-restricted pathogens transmitted in a persistent manner by hoppers (Homoptera: Auchenorrhyncha) and psyllids (Homoptera: Sternorrhyncha) (28). Their classification has been based on sequence homologies of the 16s rRNA gene (41, 19), but recently rules have been established to define a "*Candidatus* Phytoplasma species" level (23). Phytoplasmas are associated with diseases in many wild and cultivated plant species belonging to different families and cause economically important epidemics world-wide (28, 41).

The colonization of the plant by the phytoplasmas depends on the season, organ, host and pathogen species, and results in different symptoms due to complex interference with the host physiology (32). Phytoplasmas represent a major threat to the cultivation of economically important species, such as fruit trees, palms and grapevines, but efficient diagnosis is by no means trivial. Nevertheless sensitive and accurate diagnosis of these microorganisms is a prerequisite for the management of phytoplasma-associated diseases.

Molecular diagnosis

Phytoplasmas are difficult to detect due to their low concentration especially in woody hosts and their erratic distribution in the infected plants. Following their discovery (10) different approaches, such as electron microscopy observations, staining of the pathogen nucleic acid with DNA-specific dyes, grafting on indicator plants, have been used to detect phytoplasmas in symptomatic plants, usually with very poor results. Polyclonal and monoclonal antisera have also been developed for easy and inexpensive diagnostic purposes in large scale screening, but serological-based diagnosis lacks sensitivity especially when the pathogen titre is low. In the last 15 years major efforts have been made to develop nucleic acid-based tools, such as hybridization probes and PCR reagents. Phytoplasma diagnosis is now routinely done by nucleic acid-based techniques, mainly PCR.

Different protocols for total DNA extraction have been reported for the detection of these plant pathogens (1, 8, 17, 38). The success of each protocol depends on the plant host species (5, 15) as well as on the sampling procedures (36) or storage conditions of collected samples (4).

Total DNA extraction is usually the first stage in the diagnosis of phytoplasmas. Most laboratories use a phytoplasma enrichment procedure for this purpose. This is a time-consuming procedure indispensable to obtain repetitive results from field collected woody plants, such as grapevine. In some cases, such as the detection of phytoplasma diseases in several cactus species, a proteinase step must be included to obtain DNA preparation of an acceptable quality for further analysis. In insect vectors, possibly due to the high titre of the bacteria, diagnostic PCR sometimes produces acceptable results even when total DNA is prepared with a quick boiling procedure (16, 33). Recently a protocol has been proposed which includes a rapid crushing of the sampled tissue in an extraction buffer, followed by a reverse transcription-PCR to detect grapevine phytoplasmas (unpublished results).

Sequence information on the phytoplasma DNA became available after the successful isolation and characterization of the pathogen DNA from infected *Oenothera hookeri* leaf tip cultures (40). The ribosomal RNA operon became the preferred target for sequencing (29) and primers were identified in different positions of the ribosomal RNA operon to amplify phytoplasma-specific fragments from total DNA of infected plants and vectors (9, 18, 20, 21, 27, 30, 34, 43). In the meanwhile comparison of the phytoplasma-specific 16SrRNA gene sequences and the variable 16S-23S intergenic regions of phytoplasmas belonging to different strain clusters provided new information for the development of group-specific primers for a quicker preliminary characterization of the pathogen. Since phytoplasmas occur in low concentrations in the host tissues and their number is subject to seasonal fluctuations, especially in woody hosts, and even the presence of PCR inhibitor compounds in the extracts can vary throughout the year (31), in most cases a single PCR is not enough to amplify visible phytoplasma-specific bands. It is now widely accepted that diagnosis of

these pathogens is achieved with a nested PCR approach. In most laboratories, a first PCR reaction is performed using phytoplasma-specific universal primers then a nested one, driven with group-specific primers, follows to provide the first clue for the identification of the pathogen. Different combination of universal and group-specific primers have been developed. Universal primers based on the 16S rDNA gene show different sensitivity and this is possibly due to the amplicon size (usually around 1 kbp or more) as well as to mismatches in the target sequences. In some cases interferences in the diagnostic PCR have been associated to the presence of contaminating bacteria in the sample (2, 42), especially when total DNA extracts from field-collected woody samples are analysed. A further restriction length polymorphism analysis is often required to achieve the final identification of the pathogen species, even when group-specific primers are used in the nested PCR step.

Chromosomal sequences other than the ribosomal operon, such as the tuf gene (39), nitroreductase gene (24, 25), gyrase genes (7), and even sequences to which no obvious function had been predicted, have also been the target to design universal or group-specific primers. Many of these are widely used for diagnostic purposes. Other authors have designed universal or specific primers for detection, located on the sequence of plasmids hosted by phytoplasmas. This approach guarantees a high sensitivity due to the high copy number of the plasmids in the bacterial cells, but has a major drawback since it does not allow further genetic characterization of the detected pathogen. Moreover plasmids have been reported with high frequencies in some phytoplasma groups, but not in others (11) and therefore diagnosis based on extra-chromosomal DNA sequences cannot be considered as a universal approach.

Successful phytoplasma detection in insect vectors may be attained with quicker total DNA extraction procedures, probably due to the high titre of the bacteria in the insect body (33). Specific reagents and protocols have been published for the detection and identification of many phytoplasmas in potential vectors (for a review see 32). Also in these cases, endonuclease digestion of the ribosomal amplicon is required for the identification of the bacteria. This is especially true when the scope of the diagnosis is the identification of potential vector abilities of new species or also since, in many cases, the same insect can vector different phytoplasmas (4).

The most important requirements to improve diagnosis of phytoplasmas are devoted to the development of quicker, more economic and robust methods. Nested PCR protocols are extremely sensitive, but the achievement of high levels of sensitivity without the risk of false positive results that can be associated with nested PCR is also highly desirable. Real time PCR has been recently introduced as a diagnostic tool in different fields. This approach is candidate for replacing standard PCR in routine testing, due to the high sensitivity and direct reading of the results which reduce the risk of amplicon contamination and the need for a gel-based post PCR analysis. At the moment of writing, real time protocols have been used for the detection of the Apple proliferation phytoplasma (6, 14, 26), the grapevine yellows phytoplasmas (6), and the quantification of pathogen cells in chrysanthemum (32), periwinkle, and poinsettia (6) infected with different phytoplasmas.

In the last few years, several other procedures have been proposed for the analysis of the PCR amplification products from phytoplasma infected plants, including PCR-ELISA (37), PCR-dot blot (3), heteroduplex mobility assay (44, 45), 16S-23S spacer length polymorphism (35), microarray (13) and nanobiotransducer hybridization (12). Although these techniques may not have the characteristics of speed, sensitivity, and robustness of real time PCR, they are nevertheless interesting for developing future assay methods with a higher multiplexing potential, thus improving the efficiency or ability to detect multiple phytoplasmas in a single step.

It should be noted, however, that the major limitation to the development of high throughput, robust diagnostic assays for phytoplasmas remains the difficulty in developing a rapid and cost/labour effective preparation of representative nucleic acids extracts. It is well known that the phytoplasmas may be distributed very irregularly in infected plants. The most reliable diagnostic protocols, therefore, include the collection of samples as pools of subsamples taken from different parts of the individual plant to be tested. In order to reduce the amount of material to be processed usually the samples are enriched for phytoplasma and/or phytoplasma containing tissues (i.e. phloem) before proceeding with nucleic acid extraction. Although this is a lengthy step, its suppression would lead to the occurrence of an unacceptable number of false negatives. Recently a diagnostic protocol has been developed for the diagnosis of Flavescence dorèe phytoplasma (FDP) in field-collected symptomatic grapevines. The method is based on one tube reverse-transcription PCR (RT-PCR) on 16S ribosomal RNA of the phytoplasma from whole leaf sap (unpublished). The protocol has the potential advantages of RT-PCR as a rapid, reliable and sensitive diagnostic tool for FDP detection on for large scale grapevine screening. Moreover it also has the opportunity to be coupled to the real time PCR technology with further improvement in terms of speed and avoidance of contaminations.

Although methods to rapidly obtain phytoplasma enrichment, such as immunocapture PCR (22), have been developed, to our knowledge they have never been tested in comparative studies in order to assess whether or not they compare favourably with the conventional methods.

Due to the intrinsic characteristics of phytoplasma diseases, i.e. the low concentration and irregular distribution of the pathogens, it is foreseen that the problem of sample representativeness of the sample is the major obstacle to further boost diagnosis of these plant pathogens.

الملخص

مارشازي، كريستينا. 2006. التشخيص الجزيئي للفيتوبلازما. مجلة وقاية النبات العربية. 24: 139-142.

الفيتوبلازما كائنات لا يمكن زراعتها، وهي ممرضات تفتقر إلى جدار خلوي ومحدودة على اللحاء، وتنتقل بالطريقة المثابرة بوساطة نطاطات الأوراق ونطاطات النباتات (رتبة غشائيات الأجنحة وفصيلة Auchenorrhyncha) وأنواع البسيلا (رتبة غشائيات الأجنحة وفصيلة Sternorrhyncha) وتترافق هذه الكائنات مع أمراض تصيب عديداً من الأنواع النباتية البرية والمنزرعة، التي تنتمي لفصائل مختلفة، محدثة أوبئة اقتصادية مهمة في أصقاع العالم. ويتوقف استعمار الفيتوبلازما للنبات على الفصل، العضو، ونوع العائل والممرض، وتؤدي إلى أعراض مختلفة نظراً لتداخلات معقدة مع فيزيولوجية العائل. ويعدّ التشخيص الدقيق

لهذه الممرضات مهماً لإدارة الأمراض المرافقة للفيتوبلازما. والفيتوبلازما صعبة الكشف نظراً لتركيزها المنخفض، وبخاصة في العوائل الخشبية، ولتوزّعها غير المنتظم في النباتات المصابة. ويمكن حالياً تشخيص هذه الكائنات بصورة روتينية بتقاني مرتكزة على الحمض النووي، وبخاصة تقنية PCR. ويمكن الحصول على مستحضرات الحمض النووي DNA الكلي من نوعية جيّدة والغني بـــ DNA من الفيتوبلازما بتضمين خطوة إغناء بالفيتوبلازما تتطلب وقتاً كبيراً، علماً أنه تم تطوير برتوكولات أبسط باستخدام أعمدة ميكروسبن تجارية. ويمكن الوصول إلى كشف الفيتوبلازما بنجاح في النواقل الحشرية بإجراءات استخلاص أسرع للـــ DNA الكلي، وقد يكون ذلك عائداً إلى معدل عال من البكتيريا في جسم الحشرة. وقد تم تحديد بادءات عامة متخصصة بالفيتوبلازما مثل 16SrRNA و 16S-23S للفيتوبلازما المنتمية إلى مجاميع سلالات مختلفة. وتعدّ البادئات المرتكزة على تتالي الريبوزومات الأكثر استخداماً في التشخيص الروتيني للفيتوبلازما. كما تم أيضاً استهداف بادئات شائعة وأخرى خاصة بمجموعة معينة لتتالي مورثات أخرى، ولتتالي بدون أي وظيفة ولتتالي البلازميدات الموجودة في الفيتوبلازما. ويتضمن التشخيص الروتيني عادة استخدام PCR العكسي. كما تم حديثاً اقتراح تقنيات أخرى مثل RT-PCR, real time PCR, PCR-ELISA وغيرها.

كلمات مفتاحية: تعريف، تشخيص، RT-PCR، Real time PCR، PCR-ELISA، PCR-dot blot.

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