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Highly specific PCR-diagnosis to determine *Pseudomonas solanacearum* strains of different geographical origins

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Abstract Using a PCR-based assay with highly specific primers, we were able to clearly identify all of 28 different *Pseudomonas solanacearum* strains, whereas none of the other bacteria tested gave a cross reaction. The PCR sensitivity in standard dilution experiments of pure strains was in the range of 10 to 100 cells. The assay was also investigated for its suitability in routine diagnosis of potato tubers and tomato plants inoculated with various amounts of *P. solanacearum*; it reached a sensitivity of 10^3 cells per specimen. The region between primers PS96H and PS96I was sequenced for the first time and aligned. A total of 17 *P. solanacearum* strains have been sequenced, resulting in six different sequence groups. When the variable sequence was analyzed, a high correlation between point mutations and geographical origin of the *P. solanacearum* strains was revealed. The PCR assay described in this study combined with automatical sequencing of the amplificated region provides a powerful tool for the epidemiology of *P. solanacearum*.

Key words PCR · *Pseudomonas solanacearum* · Phytopathic

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Introduction

The gram-negative bacterium *P. solanacearum*, causative agent of bacterial wilt, is one of the economically most important and widely spread bacterial crop pathogens (Seal et al. 1992). *P. solanacearum* has a wide host range of about 200 diverse plants from 44 plant families, including such important crops as banana, potato and tomato (Buddenhagen and Kellmann 1964; Hayward 1991, 1994). Since 1978 *P. solanacearum* has been classified as an EPPO A2 quarantine organism (OEPP/EPPO 1978). The bacterial wilt disease is widely distributed in tropical, subtropical and warm temperate regions and is a major constraint in the culture of numerous crop plants (Hayward 1991). However, in the past decade an increasing number of *P. solanacearum* infections in potato or tomato have also been observed in cooler climatic zones like Northern Europe (Müller 1996). There are now official reports of *P. solanacearum* from Belgium, England, France, Italy, the Netherlands, Austria, Portugal and Sweden (Müller 1996). The infections described in Europe are caused solely by race 3, biovar 2 of *P. solanacearum*, which has adapted to the cooler climate (Swanepoel 1990). While this race has the narrowest of host ranges (Smith et al. 1995) and is infectious only in potato and tomato plants, it is occurring also in latency on *Solanum dulcamara* (Müller 1996). In 1995 in the Netherlands, *P. solanacearum* infections reached a nearly epidemic level resulting in a US $30 million loss in potato harvest (Anonymus 1996). Due to this steadily increasing occurrence of *P. solanacearum* in Europe, all imported potato charges from third countries and also the seed potato charges in Europe have to be tested for *P. solanacearum*, resulting in an increasing number of specimens to be examined. Up to now specimens have usually been tested by an immunofluorescence assay (Anonymus 1990) using different antisera against *P. solanacearum*. This test often exhibits false positive or

unclear results, which then must be examined further by a time-consuming bio-assay and plating of the bacteria on selective media. It is also very important to be able to detect latent *P. solanacearum* infections because of the pathogen's ability to persist for long periods in a saprophytic life style in pure soil without any host plant, thereby providing an inoculum for subsequent infection of newly growing seeds (Hayward 1991; Schell 1996). Consequently, a great deal of effort has been expended to develop an inexpensive alternative diagnostic tool which is capable of detecting even minute amounts of *P. solanacearum* in crude potato extracts unambiguously and quickly. For this purpose the polymerase chain reation (PCR) is a strong candidate, and Seal et al. (1992, 1993) designed two different primer pairs for the detection of *P. solanacearum.* The pair Y2/OLI1 corresponds to a 16S rRNA region and is not very specific for *P. solanacearum* because the primer Y2 belongs to a region of the 16S rRNA conserved in many bacteria (Young et al. 1991; Seal et al. 1993). The other pair, PS96H and PS96I, was found by subtractive hybridization experiments and is described as being highly specific for *P. solanacearum* (Seal et al. 1992), which we were able to confirm in our investigations.

In this study we used the highly specific primer pair PS96H and PS96I for the detection of 28 different pure *P. solanacearum* strains in a modified PCR assay. Furthermore, we tested the suitability of the PCR assay for detection of *P. solanacearum* in crude extracts from tomato plants and potato tubers. For the first time we report on the sequence of the region between the two primers PS96H and PS96I from 17 *P. solanacearum* strains and their alignment into six different groups.

Materials and methods

Bacterial strains

These are listed in Table 1.

Infection of tomato plants

A 500-µl aliquot of an overnight culture from purified bacteria, (approximately 10^9 cells of *P. solanacearum*) was injected into all of the axils of the leaves and at ten different locations distributed all over the stem of a 3- to 4- week-old tomato plant (*Lycopersicon esculentum* cv 'Rutgers').

Preparation of PCR-amplifiable material

Bacterial cells

Bacterial cells from all of the strains investigated in this study were transferred directly from culture plates into reaction tubes, and in the first PCR step heated for 3 min at 96°C. The initial heating step of PCR was always sufficient for amplification of the DNA fragment between PS96H and PS96I out of pure bacteria strains.

Plant material

To examine the applicability of the PCR assay in the case of natural plant material, we extracted stem parts of tomato plants and heel end cores of inoculated potato tubers.

Tomato plants A 0.5-cm-thick disk of the stem-basis was cut out and ground in sealed plastic bags filled with 5 ml phosphate buffer (50 m*M*, pH 7.0). After a first centrifugation of 10 min at 180 *g* and 10°C the supernatant was subjected to a second centrifugation of 15 min at 7.000 *g* and 10°C. The resulting pellet was resuspended in 3 ml phosphate buffer (10 m*M*, pH 7.2), and 2 µl were taken for PCR amplification.

Potato tubers Heel end cores of 200 healthy potato tubers were extracted as described previously, and 1 ml of the potato suspension was inoculated with various amounts of *P. solanacearum* cells (strain GSPB 1958) resuspended in water. A 2-µl aliquot of the inoculated potato tuber suspension was subjected to PCR amplification.

PCR amplification

All *P. solanacearum* strains (Table 1) were tested by PCR with the primer-pair PS96H and PS96I (Seal et al. 1992) which corresponds to a 148-bp-long genomic sequence of *P. solanacearum.* PCR amplifications were performed using a water-cooled thermal cycler (Autogene II, Grant). Reaction volumes were 50 µl and contained 1 × PCR buffer (Qiagen), 0.2 m*M* of each dNTP, 0.5 units *Taq* polymerase (Qiagen), 0.3 µ*M* of each primer and 2 µl of extracted tomato or potato plant material or an undetermined number of bacterial cells from culture plates. Each reaction was initially heated for 3 min at 96°C and then cycled 35 times through denaturation (94°C for 25 s), annealing (64°C for 30 s) and elongation (72°C for 30 s), finished by a final period of 5 min at 72°C. Samples of the reaction mixture (5–10 µl) were examined by electrophoresis using agarose gels (2% w/v, GIBCO), and these were subsequently visualized by ethidium bromide staining.

Dilution experiments To determine the sensitivity of the PCR assay, we boiled serial dilutions of pure *P. solanacearum* strain GSPB 1958 for 10 min in 50 m*M* NaOH, 1% SDS and then subjected them to PCR for 40 cycles as described. The initial number of bacteria for each dilution series was determined by counting the bacterial cells in a Thoma chamber.

Sequencing of the PCR products

Before sequencing, the PCR product was purified twice using the "High Pure PCR Purification Kit" from Boehringer Mannheim. All sequencing reactions were performed with the "Dye Didesoxy Terminator Kit" from Applied Biosystems as described by the manufacturer's protocol. Gel running and gel data analysis were performed with an automatic sequencer type A 373 from Applied Biosystems. The resulting sequence was analyzed with DNASIS (Pharmacia), BLAST (EBI) and aligned with MULTALIN (Corpet 1988).

Results

PCR detection of 28 different pure *P. solanacearum* strains

All 28 available *P. solanacearum* strains were tested by PCR with primers PS96H and PS96I as described.

Table 1 Listing of all tested bacterial strains

Number	Bacterial strains	Country of origin	Sequence group
	P. solanacearum		
1	PD 1453 (accession no: Y11126)	Columbia	3
2	PD 1608	Netherlands	1
3	PD 1655 (accession no: AJ001327)	USA, Florida	5
4	PD 2762	Netherlands	1
5	PD 2763	Netherlands	1
6	GSPB 1958 (accession no: Y11125)	Egypt	1[a]
7	GSPB 1960	Sweden	1
8	GSPB 2113	Columbia	3
9	GSPB 2121	Indonesia	6
10	GSPB 2124	Unknown	1
11	GSPB 2126	Unknown	n.s.[b]
12	GSPB 2128	Australia	n.s.
13	DSM 1993 (accession no: Y11127)	Mauritius	6
14	DSM 9544	USA	5
15	DSM 50905 (accession no: AJ001328)	Costa Rica	2
16	IPV-Bo 2893	Italy	1
17	2958 (accession no: AJ001329)	Guatemala	4
18	3863	France	n.s.
19	3864	Egypt	n.s.
20	3865	France	n.s.
21	3866	France	n.s.
22	PS 1-95	Egypt	1
23	196/96-1	Egypt	n.s.
24	Ä93	Egypt	n.s.
25	Ä95	Egypt	n.s.
26	96/112/1a	Egypt	n.s.
27	96/157/3a	Egypt	n.s.
28	NL	Netherlands	1
	Other pseudomonads		
29	DSM 2089 *P. syzygii*		
30	DSM 2093 *P. syzygii*		
31	DSM 6297 *P. pickettii*		
32	DSM 6519 *P. eutropha*		
33	GSPB 2117 *P. fluorescens*		
	Other bacteria		
34	DSM 1025 *Erwinia carotophora* subsp. *atroseptica*		
35	DSM 1386 *Clavibacter michiganensis*		
36	DSM 1488 *Clavibacter michiganensis*		

[a] Strain used as reference for group 1
[b] Not sequenced

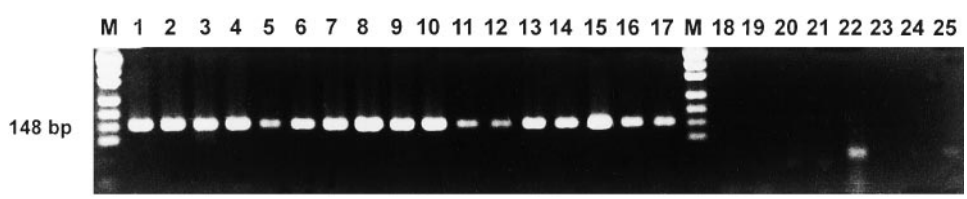

Fig. 1 Result of a PCR with the *P. solanacearum*-specific primers PS96H and PS96I. Only the 17 *P. solanacearum* strains exhibited a PCR product with the specific length of 148 bp. *Lanes 1–17* 17 different *P. solanacearum* strains, *18 P. syzygii* 2089, *19 P. syzygii* 2093, *20 P. pickettii* 6297, *21 P. eutropha* 6519, *22 P. fluorescens* 2117, *23 Erwinia carotophora* sub. *atroseptica* 1025, *24 Clavibacter michiganensis* 1386, *25 Clavibacter michiganensis* 1488, *M* DNA-length marker 8 (Boehringer Mannheim)

Furthermore, 2 different strains of *P. syzygii*, 1 of *P. pickettii*, *P. eutropha*, *P. fluorescens*, *Erwinia carotophora* subsp. atroseptica and 2 different *Clavibacter michiganensis* strains were tested in the PCR-assay.

In Fig. 1 the 148-bp-long PCR product of 17 out of the 28 strains is shown. All of the *P. solanacearum* strains could be clearly detected by PCR (Fig. 1, lanes

1–17), whereas neither the 5 related *Pseudomonas* strains (lanes 18–22) nor the other bacteria species (lanes 23–25) exhibited a specific PCR product.

PCR sensitivity and suitability of the assay for crude plant extracts

For determining the sensitivity of the modified PCR assay, which is originally described with 10–100 cells (Seal et al. 1992), we performed tenfold dilution series of the pure *P. solanacearum* strain GSPB 1958 ranging from 10^4 to zero cells. Five microliters from each dilution step was subjected to 40 PCR cycles.

In Fig. 2 the PCR product of dilution series from *P. solanacearum* strain GSPB 1958, ranging from 10^4 to zero cells (lanes 1–6), is shown. According to the literature we were able to detect at least 10 to 100 cells with the modified protocol (Fig. 2, lanes 1–4).

To investigate the suitability of the PCR assay for detection of *P. solanacearum* in plant material, we used a crude extract of stem disks from *P. solanacearum*-infected tomato plants and a suspension of heel end cores from potato tubers inoculated with *P. solanacearum* cells as described.

All infected tomato plants (Fig. 3, lanes 1–4) and potato tubers (lanes 5–8) inoculated with *P. solanacearum* strain GSPB 1958 showed the same specific PCR product as the pure strain (lane 9). The mock-inoculated tomato plant (lane 10) and the negative control (lane 11) gave no PCR product. We also performed dilution series with crude plant extracts, reaching a sensitivity of 10^3 cells (data not shown). This reduced sensitivity compared with the pure strain is most probably caused by the crude plant extract which contains various PCR inhibitors (Seal 1995).

Sequencing and relationship between the sequence and geographical origin of the *P. solanacearum* strains

The sequence between the used primers PS96H and PS96I was 94 bp long and showed a number of point mutations between the different strain groups. In total we sequenced 17 *P. solanacearum* strains (Table 1) and aligned their sequences into six different groups (Fig. 4) in which all members were identical. We used strain GSPB 1958 as reference for group 1 with 8 other strains showing the same sequence. The six individual groups differed in one up to six point mutations over 94 bp, which translates into a 1.1–6.4% difference.

In Fig. 4 the six different sequence groups of *P. solanacearum* strains are shown. The point mutations between their sequences are highlighted in gray boxes. The groups are listed regarding their descending homology compared to reference group 1. The sequences of members from all six groups have been

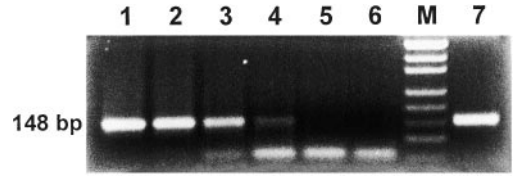

Fig. 2 PCR with primers PS96H and PS96I using dilution series from the pure *P. solanacearum* strain GSPB 1958. A specific PCR product was detectable from at least 10^1 bacterial cells. Lane *1* 10^4, *2* 10^3, *3* 10^2, *4* 10^1, *5* 1, *6* 0 cells of *P. solanacearum* strain GSPB 1958, *7* 2 µl overnight culture of *P. solanacearum*, *M* DNA-length marker 8 (Boehringer Mannheim)

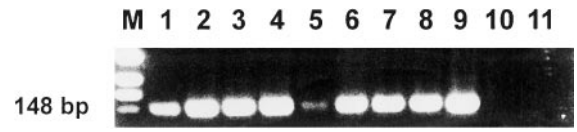

Fig. 3 Result of a PCR-detection of *P. solanacearum* in crude plant extracts using the primers PS96H and PS96I. Only tomato plants or potato tubers infected with *P. solanacearum* exhibited a specific PCR product. *Lanes 1–4* PCR with 2 µl of crude extract from 4 different tomato plants, *5–8* PCR with 2 µl of crude extract from 4 different potato tuber heel end cores, *9* PCR with 2 µl overnight culture of *P. solanacearum*, *10* PCR with 2 µl of crude extract from a mock inoculated tomato plant, *11* PCR with 2 µl sterile water; *M*: DNA-length marker 8 (Boehringer Mannheim).

submitted to the EMBL database and are available under their accession numbers (see Table 1).

Remarkably, there is a clear relationship in the sequenced strains between the sequence and their geographical origin (Fig. 5). All 6 European strains of group 1 were sequence-identical with the 2 Egyptian strains but appeared after 1989 scattered over the European continent presumably introduced by imported potato seeds from Egypt (Müller 1996). The 2 members of group 3 were Columbian strains, which show clear differences to the strain of group 2 from nearby Costa Rica and the only member of group 4 from Guatemala. The 2 sequenced strains of North American origin representing group 5 were also different from Central and South American strains. Group 6 also consists of 2 identical strains which derived from Indonesia and Mauritius. In this case it is not known whether 1 of the strains was introduced by seeds or developed naturally.

Discussion

The original PCR assay of Seal et al. (1992), modified here for cycle conditions, is a very specific and suitable test for *P. solanacearum* infections of solanaceous plants. In our investigation we found no cross reactions in PCR using the primer pair PS96H and PS96I with any other tested bacteria or plant material. In the dilution series we could detect 10–100 cells per PCR in

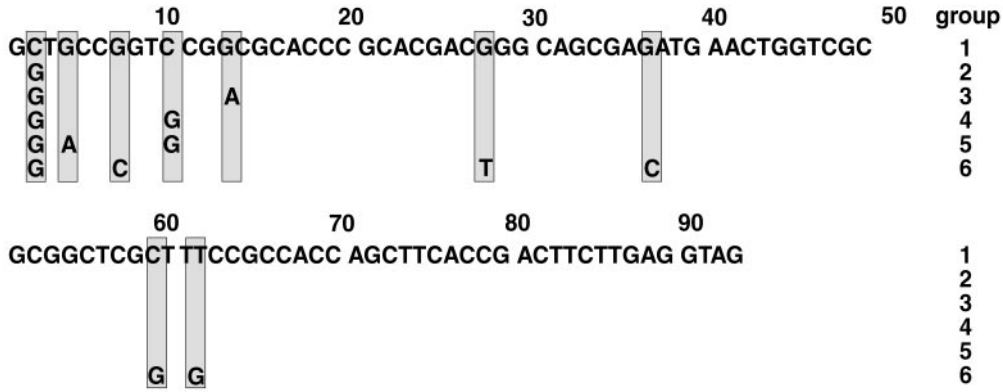

Fig. 4 Alignment of the six *P. solanacearum* sequence groups differing in the region between PCR primers PS96H and PS96I. The six sequence groups derived from eight different countries show one to six point mutations. Only the 94-bp-long sequence of group 1 is completely shown; the point mutations in which the other groups differ from group 1 are *highlighted* in *gray boxes*. *1* Egypt/Europe, *2* Costa Rica, *3* Columbia, *4* Guatemala, *5* North America, *6* Indonesia/Mauritius.

standard experiments with pure strains. The assay also works well under field conditions. Using the same extraction protocol as described for routine diagnosis in the European plant protection institutes (Quarantine pests for Europe 1992) we always obtained unambiguous PCR amplification products. The reduced sensitivity of the PCR assay (10^3 cells) with crude plant extracts is very probably caused by PCR inhibitors. These inhibitors could be avoided; for example by using Immuno-Capture-PCR with *P. solanacearum*-specific antibodies or by a DNA extraction protocol preceding the PCR. Unfortunately, such modifications would complicate the diagnosis procedure, leading to an increase in time required and costs. In further tests for solving the inhibitor problem one must consider these costs very carefully, because the assay must be cheap and also applicable in third-world countries.

The sequencing of the unknown region between PS96H and PS96I gave very interesting results. In comparison to the commonly used 16S rDNA sequence which is highly conserved, this region is very variable, showing 1.1–6.4% sequence divergency between the individual strains from different geographical origins. Consequently, this region is very suitable for highly specific diagnosis of *P. solanacearum* with PCR and the primers PS96H and PS96I. In less-related bacteria this sequence either does not exist or it must be so far evolved that one or both PCR primers are unable to bind under normal annealing conditions. Furthermore, the sequence of the resulting PCR product can be used for determining the geographical origin of each strain which causes the infection. For this purpose we highly recommend sequencing the region between PS96H and PS96I from as many worldwide distributed strains as are available so as to build up a reference database. On

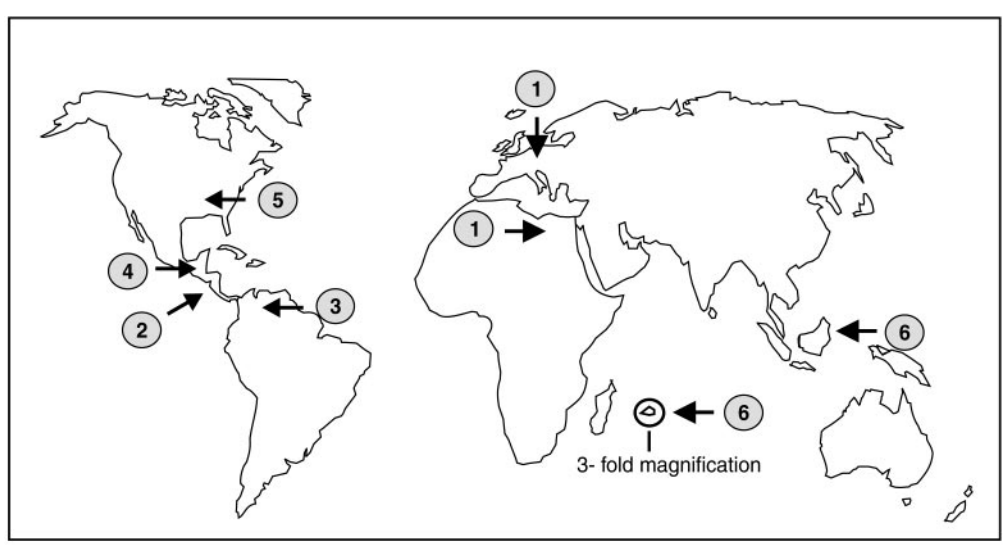

Fig. 5 Worldwide geographical distribution of the six different sequence groups of *P. solanacearum*. The origin country of the *P. solanacearum* strains from each of the six sequence groups is marked with a *black arrow*. The isle Mauritius is shown in threefold magnification for better visibility. Each group consists of one to nine different strains. *1* Egypt/Europe, *2* Costa Rica, *3* Columbia, *4* Guatemala, *5* North America, *6* Indonesia/Mauritius

the basis of these databases each new isolate could be aligned quickly, and its origin could very probably be determined. Although the known sequence is relatively short (94 bp), up to now all of the sequenced strains from different geographical origins could be distinguished. In further analyses, the flanking regions should be isolated and characterized, for example by Inverse-PCR, to initiate epidemiological investigations on the occurrence and spread of different *P. solanacearum* strains worldwide.

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