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Novel T-RFLP method to investigate six main groups of 2,4-diacetylphloroglucinol-producing pseudomonads in environmental samples

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ABSTRACT

Strains of fluorescent pseudomonads producing 2,4-diacetylphloroglucinol (DAPG) are involved in the protection of plant roots against soil-borne plant pathogens. Recently, a multilocus sequence analysis of a world wide collection of DAPG-producers led to the identification of six main groups (A–F). In this study a T-RFLP method based on the *phlD* gene was developed to efficiently identify the members of these six groups in environmental samples. A combination of six restriction enzymes was identified which leads to group specific terminal fragments (T-RF). The detection limit of the *phlD*-T-RFLP method was determined for the two *P. fluorescens* strains F113 (group B) and CHA0 (group F) in rhizosphere samples and was found to be 5×10^3 CFU/g and 5×10^4 CFU/g respectively. *PhlD*-T-RFLP and *phlD*-DGGE analysis of wheat and maize root samples from greenhouse and field revealed similarly the presence of multilocus groups A, B and D. However, they were more frequently detected with *phlD*-T-RFLP. Additionally, groups C and F were detected in greenhouse samples but only by *phlD*-T-RFLP and not by *phlD*-DGGE. In conclusion, the new *phlD*-T-RFLP method proved to be a fast and reliable method to detect strains of the six main groups of DAPG-producers in environmental samples with an improved detection limit compared to *phlD*-DGGE.

1. Introduction

Root-colonizing bacteria belonging to the group of fluorescent pseudomonads are known to be involved in the protection of plant roots against soil-borne plant pathogens. This beneficial plant–bacteria interaction is often based on the production of antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG). Strains of *P. fluorescens* which produce DAPG have been recovered from the rhizosphere of many plants (De La Fuente et al., 2004, Keel et al., 1996, McSpadden Gardener et al., 2000, Thomashow and Weller, 1995) worldwide and their contribution to the suppressiveness of certain soils against soil-borne plant pathogens has been shown in several studies (de Souza et al., 2003, Raaijmakers and Weller, 1998, Stutz et al., 1986, Weller et al., 2002). Due to their important role in disease suppression, their genotypic and phenotypic diversity has been extensively studied. Different molecular methods like restriction analysis of 16S rRNA genes (ARDRA) (Keel et al., 1996, McSpadden Gardener et al., 2000, Picard et al., 2000), repetitive sequence-based PCR (REP-PCR) (Landa et al., 2006), random amplified polymorphic DNA analysis (RAPD) (Mavrodi et al., 2001) and *phlD* gene restriction fragment length polymorphism (RFLP) (Mavrodi et al., 2001, Ramette et al., 2001, Wang et al., 2001) were used so far revealing variable degrees of diversity among DAPG-producing pseudomonads. The *phlD* gene is essential for the DAPG production (Fenton et al., 1992, Keel et al., 1992) and conserved among DAPG-producing *P. fluorescens* strains. More recently the diversity of DAPG producing *P. fluorescens* strains has been characterized by denaturing gradient gel electrophoresis (DGGE) or sequence analysis of the *phlD* gene (Bergsma-Vlami et al., 2005, De La Fuente et al., 2006, Frapolli et al., 2008). With the exception of DGGE all other methods depend on the isolation of *phlD*$^+$ *P. fluorescens* strains from the environment and cultivation on selective media prior to characterization. This cultivation step may create a bias towards the most predominant or easily cultivable genotypes and therefore distort the results. For this reason culture-independent methods using DNA extracts from environmental samples are more favorable to assess the diversity of DAPG-producing pseudomonads. Whereas the two known DGGE methods (Bergsma-Vlami et al., 2005, Frapolli et al., 2008) do not have this bias they both have practical limitations. As the method of Bergsma-Vlami et al. (2005) uses two different primer sets for the amplification of the *phlD* gene, it is very time consuming. The method of Frapolli et al. (2008) uses only one primer set, but the detection limit of their method is quite high, 10^5 CFU/g soil for Q2–87 and even 10^7 CFU/g soil for CHA0. There is a need for faster and more sensitive methods to investigate DAPG-producing pseudomonads.

Such a method could be based on terminal restriction fragment length polymorphism (T-RFLP) analysis. T-RFLP is a culture-independent method to effectively assess the diversity of a microbial community and is one of the most commonly used molecular methods for community

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structure determination (Marsh, 2005, Nocker et al., 2007, Schutte et al., 2008). It is a fast high-throughput fingerprinting method, which because of its relative simplicity has been applied for the analysis of different systems, e.g. fungal ribosomal genes, bacterial 16S rRNA genes, archael 16S rRNA genes and also functional genes (Schutte et al., 2008). The T-RFLP method identifies species diversity based on comparison of the length of the terminal restriction fragments (T-RF). It is considered to be more sensitive and faster than DGGE (Moeseneder et al., 1999, Nunan et al., 2005).

Recently, a multilocus sequence analysis of a worldwide collection of DAPG producing *P. fluorescens* strains identified six main phylogenetic groups (A–F) which could taxonomically even correspond to six different species (Frapolli et al., 2007). 16S rRNA sequence analysis showed that groups A–E belong to the *P. fluorescens* complex and that groups A and C are closely related to *Pseudomonas aurantiaca* and *Pseudomonas brassicacearum*, group B to *Pseudomonas kilonensis*, group D to *Pseudomonas thivervalensis* and group E to an uncharacterized species in the vicinity of the *Pseudomonas synxantha* cluster (Frapolli et al., 2007). Group F, however, which contains very closely related strains and which was already defined as a separate group of *phlD*-producers (ARDRA 1 group) by Keel et al. (1996)) does not belong to the *P. fluorescens* complex as shown by Frapolli et al. (2007) and Ramette et al. (2003) and most probably corresponds to a new species. Also biocontrol traits can be different between these groups. Group F for example produces a second potent antimicrobial metabolite, pyoluteorin, which is not produced by groups A–E. So far, DAPG producers can be assigned to one of these six multilocus groups/species by sequencing some housekeeping genes according to Frapolli et al. (2007). However, a cultivation independent method for the easy detection and differentiation of these six important groups of biocontrol bacteria in environmental samples is missing.

The main objective of this study was to develop a T-RFLP method which can be used to identify these six phylogenetic groups described by Frapolli et al. (2007) in environmental samples. The new T-RFLP approach was then further used to assess the diversity of *phlD*$^+$ pseudomonads with respect to the six phylogenetic groups in the rhizosphere of maize and different wheat varieties grown in natural soil. Finally, we compared the T-RFLP method with a *phlD*-DGGE approach by analyzing the same rhizosphere samples with the two methods.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

The *Pseudomonas fluorescens* strains used in this study are listed in Table 1. Each strain was grown at 27 °C in 10 ml liquid King's medium B (King et al., 1954) for 24 h on a rotary shaker (Lab-Therm, Kühner, Switzerland) at 120 rpm.

2.2. PCR amplification of the PhlD gene

The *phlD* gene of reference pseudomonads was amplified using DNA from lysed pure and mixed cultures. PCRs were carried out in 20-µl reaction mixtures, containing 5% dimethylsulfoxide (Fluka, Switzerland), 5% bovine serum albumin, 1× ThermoPol Buffer (New England Biolabs, Inc., USA), 100 µM of each dNTP, 0.4 µM of each forward (B2BF, 5′-ACCCACCGCAGCATCGTTTATGAGC-3′) and reverse (BPR4, 5′-CCGCCGGTATGGAAGATGAAAAAGTC-3′) primer (B2BF and BPR4 are *phlD*-specific primers, McSpadden Gardener et al., 2001), 0.15 µl Taq DNA-Polymerase (5000 U/ml, New England Biolabs, Inc., USA)

Table 1
Origin, biocontrol properties and corresponding multilocus groups of the 40 DAPG-producing reference *P. fluorescens* strains used in this study.

Origin	*P. fluorescens* strains (multilocus group by Frapolli et al., 2007)	Biocontrol activity[a]	Reference	*PhlD* sequence accession number
Texas, USA	**Pf-5 (F)**[b]	Cotton (Pu, Rs), cucumber (Pu)	Howell and Stipanovic, 1980	AF214457.1
Oklahoma, USA	PF (F)	Wheat (St)	Levy et al., 1992	NA[c]
Quincy, USA	**Q2–87 (C)**	Wheat (Ggt)	Vincent et al., 1991	PU41818
	Q1–87 (C), Q12–87 (C), Q13–87 (C), **Q37–87 (B)**, Q86–87 (C)	Wheat (Ggt)	Keel et al., 1996	AY928631.1,NA, NA, AY928641.1, EF554356.1
	Q65c-80 (A)	Wheat (Ggt)	Harrison et al., 1993	AJ278807.1
El Batan, Mexico	F96.26 (E)	Tomato (FORL)	Wang et al., 2001	EF554357.1
Ireland	**F113 (B)**	Sugar beet (Pu)	Shanahan et al., 1992	AJ278811.1
Morens Switzerland	**CHA0 (F)**	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	Stutz et al. 1986	AJ278806.1
	CM1′A2 (A)	Cucumber (Pu, Ps), cotton (Rs)	Fuchs and Défago, 1991	AJ278808.1
	K93.2 (B)	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	EF424746.1
	P12 (B)	Tobacco (Tb), cucumber (Pu)	Keel et al., 1996	EF424743.1
	P97.38 (E)	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	EF424749.1
	TM1A3 (A), TM1B2 (A), C*1A1 (A)	Cucumber (Pu), cotton (Rs)	Fuchs and Défago, 1991	NA, NA, NA
	S7–46 (A), S8–110 (B), S8–130 (B), S8–151(A), **C10–181 (B)**, C10–186 (B)	Tobacco (Tb)	Ramette et al., 2003	EF554346.1, EF554348.1, EF554349.1, EF554350.1, EF554332.1, EF554333.1
Albenga, Italy	**PILH1(D), PITR2 (D)**	Cucumber (Pu), tomato (FORL)	Keel et al., 1996	AJ278810.1, AJ278809.1
	K93.52 (D)	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	EF554351.1
West Russia	P96.25 (A)	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	NA
Tallinn, Estonia	**F96.27 (E)**	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	EF424744.1
Punakha Wangdi, Bhutan	P97.1 (E), P97.6 (E), **P97.27 (E)**	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	EF554353.1, NA, EF554354.1
Mitra, Slovakia	**K94.31 (A)**, K94.37 (B), K94.38 (B)	Tomato (FORL)	Wang et al., 2001	EF424748.1, EF424745.1, EF554352.1
	K94.41 (F), **P97.30 (A)**	Cucumber (Pu), tomato (FORL)	Wang et al., 2001	NA, EF424747.1
Ghana	**PGNR1 (F)**, PGNL1 (F)	Cucumber (Pu), tomato (FORL)	Keel et al., 1996	AY928629.1, FJ012275.1

[a] Ggt, *Gaeumannomyces graminis* var. *tritici*; FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; Tb, *Thielaviopsis basicola*; St, *Septoria tritici*.
[b] Sequences of strains which are highlighted in bold were used to screen for specific restriction sites.
[c] NA, *phlD* sequence not available.

and 2 µl of template DNA. The following thermocycling conditions were used: 94 °C for 2 min 30 s, 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min (35 cycles) and 72 °C for 10 min. The presence of the amplified fragments was checked on a 1% agarose gel in 0.5% TBE buffer.

2.3. Unique restriction sites

The *phlD* gene sequences of 20 *phlD* positive *P. fluorescens strains* (strains and GenBank accession numbers of *phlD* sequences indicated in Table 1) were used to find unique restriction sites corresponding to a specific multilocus group (A–F) of DAPG producers as determined by Frapolli et al. (2007). The sequences were aligned with the Sequencer Software 4.8 (Applied Biosystems, Foster City, Ca) and scanned for unique restriction sites which allow to separate the six phylogenetic groups. Restriction enzymes were chosen corresponding to their ability to be used in a multiple digest analysis.

2.4. Restriction enzyme analysis

Lysed DNA of overnight cultures of reference *P. fluorescens* strains (Table 1) were used to test the identified restriction enzymes (Table 2) in pure and mixed samples. The *phlD* gene of pure and mixed cultures of all 40 reference bacteria were amplified as described above. The PCR products were digested for 4 h at 37 °C with single and multiple restriction enzymes in 12 µl reaction mixtures containing 5 µl of PCR product, 1.2 µl Enzyme Buffer (Fermentas, Ca) and 0.5 U of each enzyme (BspI, NspI, AseI, EcoO109I, Kpn2I and PstI). After the incubation time the enzymes were inactivated for 5 min at 80 °C and the fragments were checked on a 1% agarose gel in 0.5% TBE buffer.

2.5. T-RFLP analysis of the PhlD gene

DNA from pure and mixed cultures as well as DNA from rhizosphere samples spiked with reference strains (preparation of spiked rhizosphere samples see below) was used to amplify the *phlD* gene as described above with a labelled reverse primer BPR4. The reverse primer BPR4 was labelled at its 5-prime end with Carboxy-fluorescin (FAM) or its hexa-chloro derivative (HEX). The PCR amplification of spiked soil samples was performed with an increased annealing time (2 min) and 40 instead of 35 cycles. The PCR products were checked on agarose gels and then digested as described above. For T-RFLP analysis, 1 to 2 µl of the digest was mixed with 9 µl HiDi formamide (Applied Biosystems, Foster City, Ca) and 0.4 µl of GeneScan-600 LIZ® (Applied Biosystems, Foster City, Ca). The samples were incubated for 3 min at 96 °C and immediately put on ice prior to fragment analysis. The length of the terminal restriction fragments T-RF was analyzed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, Ca) equipped with 500 mm capillaries filled with POP-7™ polymer. The resulting data was analyzed using GeneMapper Software 4.0 (Applied Biosystems, Foster City, Ca). The Peak detection limit was set to 50 fluorescence intensity units.

2.6. Specificity of the new T-RFLP method

The specificity of the T-RFLP method was tested with lysed DNA of overnight cultures of all reference *P. fluorescens* strains (Table 1) and DNA from maize rhizosphere samples spiked with reference *P. fluorescens* strains (indicated in Table 1). Rhizosphere samples were prepared as follows: Roots of three week old maize plants grown in autoclaved field soil (Wangen 1, Switzerland; von Felten et al., 2010) were recovered and large soil aggregates were carefully removed. The samples were cut in small root fragments and homogenized for 60 s in a mixer mill (Retsch MM 300, Germany). Lysing Matrix E tubes (FastDNA Spin Kit for Soil, MP Biomedicals, Ca) were filled with 0.3 g of homogenized rhizosphere sample and spiked with 100 µl of overnight cultures of one respectively a mixture of the reference strains. The overnight cultures were adjusted to OD 0.125 which corresponds to about 10^8 of cells (for strain CHA0). Mixtures of inoculum were produced by mixing equal amounts of single strains together and adding 100 µl to the extraction tubes. The DNA was further extracted as described below and samples were analyzed using T-RFLP as described above.

In order to further validate our method we checked published *phlD* sequences from cultured strains (De La Fuente et al., 2006) and *phlD* sequences derived from a cultivation-independent approach (DGGE bands, Frapolli et al., 2008) for presence of the required restriction sites and for length of the resulting terminal fragments.

2.7. Estimation of the detection limit of the new T-RFLP method

The detection limit of the T-RFLP method was estimated in the rhizosphere of maize inoculated with 10 times dilution series (5×10^8 to 5×10^2 CFU/g rhizosphere) of *P. fluorescens* strains CHA0 and F113. The two strains were chosen due to their differences in amplification efficiencies of the *phlD* gene (McSpadden Gardener et al., 2001). Three independent replicates of each dilution step in the rhizosphere of maize were prepared and analyzed as described above.

2.8. MPN-PCR to enumerate total and DAPG-producing P. fluorescens strains

Four replicates of 20 µl overnight stored root samples were serially diluted (1:10) in 96-well microtiter plates (Greiner Bio-one GmbH, Germany) filled with 180 µl of the *Pseudomonas*-selective King's B medium supplemented with 40 µg/ml ampicillin, 13 µg/ml chloramphenicol and 100 µg/ml cycloheximide. Microtiter plates were incubated under slight agitation at 27 °C in the dark, and bacterial growth was assessed after 48 h. Of each positively assessed dilution step 10 µl were transferred into a PCR plate with 90 µl ddH_2O. Cell suspensions were lysed by heating at 99 °C for 10 min in a T-Gradient thermocycler (Biometra, Germany). *PhlD*$^+$ wells were determined by PCR amplification of 2 µl lysed cell suspension as described above. *PhlD*$^+$ and total *P. fluorescens* cell numbers per gram fresh weight were calculated using "Most Probable Number" (MPN) calculations (Briones and Reichardt, 1999, McSpadden Gardener et al., 2001).

Table 2
Abundance of total and *phlD*-positive *Pseudomonas spp.* on roots of four Swiss wheat cultivars (Frisal, Fiorina, Casana and Bobwhite S98 26) and the commercial maize hybrid DK315. Plants were grown for six weeks either in the field or in a greenhouse pot experiment using the same soil as in the field.

Plant cultivars	*Pseudomonas* spp. (log CFU/g root)		*PhlD*$^+$ *P. fluorescens* (log CFU/g root)		*PhlD*$^+$ *P. fluorescens* (% of total *Pseudomonas* spp.)	
	Greenhouse	Field experiment	Greenhouse	Field experiment	Greenhouse	Field experiment
Casana	7.65 ab*	8.12 a	6.86 bc	5.79 a	23.72 a	2.35 a
Fiorina	8.04 a	7.34 a	6.97 ab	4.82 a	11.99 a	0.31 a
Frisal	7.31 b	8.10 a	6.55 cd	5.56 a	20.71 a	0.71 a
Bobwhite S98 26	7.81 ab	7.55 a	7.33 a	5.50 a	23.00 a	2.76 a
DK315	7.37 b		6.25 d		13.43 a	

* Different letters indicate significant differences between population levels within the same experiment using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

2.9. Greenhouse experiment

The conventional maize hybrid DK315 (Monsanto Co.) and the Swiss spring wheat cultivars Casana, Fiorina, Frisal, and Bobwhite SH98 26 were grown in pots filled with an agricultural field soil (details below) collected from the top layer (0–30 cm depth) at the research station Agroscope Reckenholz (Switzerland). The soil was air dried (20 °C), sieved (mesh size 1 cm) and stored at 8 °C until use.

Seeds of maize and wheat cultivars were surface sterilized in 2% NaClO (vol/vol) for 30 min and washed several times with autoclaved ddH_2O. Seeds were germinated on water agar plates (12 g agar in 1000 ml ddH_2O) for two days at 24 °C in the dark. Three seedlings per cultivar were planted in a pot (10.5 cm diameter) filled with Reckenholz soil. Four replicates per cultivar were prepared and arranged in a completely randomized design in the greenhouse. The plants were grown for six weeks with a relative humidity of 70% and a day–night cycle of 16 h of light at 22 °C and 8 h of darkness at 18 °C. The plants were watered twice a week with 100 ml of ddH_2O and once a week a commercial N–P fertilizer (Wuxal, Maag, Switzerland) was added to the irrigation water.

After six weeks, plants were removed from their pots and the root-adhering soil was gently removed. In a next step the roots were rinsed on a sieve (mesh size 1 cm) with water until no soil particles were visible. After the roots were shortly dried on paper tissues (Kleenex) and the fresh weight was determined. Each root sample was then placed in a 100 ml Erlenmeyer flask filled with 50 ml sterile 0.9% NaCl solution and stored overnight at 4 °C. These samples were then shaken for 30 min on a Mini-shaker (Kühner, Switzerland) at 350 rpm and four replicates of 20 µl of the root suspension were used for MPN analysis. The remaining root suspension including the roots was stored at −20 °C for total DNA extraction.

2.10. Field samples

Root samples of the wheat lines Casana, Fiorina, Frisal, and Bobwhite were taken from a field experiment performed at the research station Agroscope Reckenholz (Switzerland) in 2008 within the frame of the Swiss National Foundation research program NRP59. The soil type was classified as loamy sand (pH 7–7.2) with an organic matter content of 2.07–2.21% (data kindly provided by Agroscope Reckenholz-Tänikon ART). The same soil was used for the pot experiments described above. Each wheat line was grown in six parallel rows in plots of 7 m × 1.32 m. Four replicate plots per line were arranged in four parallel blocks using a randomized block design. Plots received fertilizer twice (in total 6 g N/m^2 in the form of NH_4NO_3) The sampling was performed after six weeks of growth when plants were at tillering stage at 1.8–1.9 m from the left border of the plot. The root stocks of ten spikes from the third and ten spikes from the fourth row of each plot were pooled. The root-adhering soil was gently removed and samples were processed further as described above for the greenhouse experiment.

2.11. DNA extraction from root samples

The total DNA from root pieces (~0.5 g) and 50 ml root suspension (prepared as described above) were extracted using Fast DNA Spin Kit for soil (MP Biomedicals, Ca). Briefly, the frozen samples were thawed overnight at 4 °C, roots were removed and 0.5 g of root pieces were added into DNA extraction tubes. The remaining suspension was centrifuged at 3500 rpm for 20 min and 50 µl of the concentrated pellets were additionally added to the extraction tubes. Total DNA was further extracted according to the manufacturer's recommendations and the DNA was diluted to a concentration of 10 ng/µl to standardize the amount of DNA. DNA extracts were then subjected to *phlD*-T-RFLP and *phlD*-DGGE analysis.

2.12. PhlD-DGGE analysis

To determine the reliability and sensitivity of the developed *phlD*-T-RFLP approach, samples of the greenhouse and the field experiment were also subjected to *phlD*-DGGE analysis. PCR amplification of the *phlD* gene for DGGE analysis was performed as described above using forward primer B2BF containing a 40 bp GC-clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′). *PhlD*-DGGE analysis was performed as described by Frapolli et al. (2008) with small modifications. Briefly, a DCode Universal Mutation Detection System (Biorad Laboratories Inc., Ca) was used to perform *phlD*-DGGE analysis. The gels contained a double gradient ranging from 7% to 12% of acrylamide and from 30% to 60% denaturing solutions (100% of denaturing solution corresponds to 7 M urea and 40% deionised formamide). The amplified *phlD* fragments of nine reference strains belonging to different multilocus groups with known migration patterns were used as markers. Gels were run at 140 V for 14 h in preheated (60 °C) 1× TAE buffer (40 mM Tris Base, 20 mM acetic acid, 1 mM EDTA, pH 8). The gels were then stained with SYBR Gold (Molecular probes, Eugene, OR) for 40 min and visualized using a UV-illuminator. Digitalized DGGE images were analyzed using Gel Compar II (Applied Maths, NV). Dominant DGGE bands were then characterized as follows. The central part of DGGE bands were cut out using sterile pipette tips. The gel pieces were then washed with 100 µl sterile ddH_2O at room temperature for 1 h and subsequently used as template for a 40 µl PCR reaction with primers BPR4 and B2BFgc using the conditions described above. The PCR products were run on a DGGE gel to check migration and presence of a single band and then sequenced using 3130 Genetic Analyzer (Applied Biosystems, Foster City, Ca). Sequences were edited using the Sequencher software (version 4.8, Gene Codes Corporation, USA) and aligned with *phlD* sequences from known pseudomonad reference strains.

2.13. Data analysis

MPN data for total and $phlD^+$ *P. fluorescens* populations were log transformed prior to statistical analysis. Analysis of variance was performed to analyze differences between the different plant cultivars and the two experiments using Systat 12.0 software (Systat Inc., Evaston, IL). Subsequently pairwise mean comparison of the individual experiments was used to determine difference of means of the cultivars using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

DGGE patterns and the T-RFLP chromatograms were converted into binary matrices (presence vs. absence of bands or peaks for each replicate) and subsequently the results of four replicates were converted to frequency matrices (frequency of band/peak occurrence, Table 3). The frequency matrices were further used for cluster analysis based on the Euclidian distances.

3. Results

3.1. Identification of phlD restriction sites and T-RFLP analysis

A T-RFLP approach, using the specific primers B2BF and BPR4 (McSpadden Gardener et al. 2001) targeting the *phlD* gene, was developed to investigate rhizosphere samples for the presence of six main groups of DAPG-producing pseudomonads. Sequences of reference strains (indicated in Table 1) were used to screen for restriction sites which result in group specific terminal fragments. A combination of six restriction enzymes (ClaI, NspI, AseI, EcoO109I, Kpn2I and PstI) was identified which allows the discrimination of the terminal fragments of the six groups (Fig. 1) resulting in T-RF with expected sizes of 215 bp (group D), 389 bp (group C), 528 bp (group E), 539 bp (group A), 548 bp (group F) and 592 bp (group B). The enzymes were tested using DNA from lysed bacteria of pure and mixed cultures of 40 reference strains for

Table 3
Frequencies of main phylogenetic groups of DAPG producing fluorescent pseudomonads identified with T-RFLP and *phlD*-DGGE in root samples of the four Swiss wheat cultivars Frisal, Fiorina, Casana and Bobwhite S98 26 as well as the commercial maize hybrid DK315 grown in the greenhouse and under field conditions.[a]

	Multilocus groups	DGGE bands	Frisal			Fiorina			Casana			Bobwhite			DK315		
			Multilocus groups		DGGE bands	Multilocus groups		DGGE bands	Multilocus groups		DGGE bands	Multilocus groups		DGGE bands	Multilocus groups		DGGE bands
			T-RFLP	DGGE		T-RFLP	DGGE		T-RFLP	DGGE		T-RFLP	DGGE		T-RFLP	DGGE	
Greenhouse	A		1	1		1	1		1	1		1	1		1	1	
		A2			1			1			1			1			1
		A3			0.5			0.75			0.75			0.5			0.25
	B		1	1		1	1		1	1		1	1		1	1	
		B1			0.5			0.5			0.5			0.5			0
		B2			0			0			0			0.5			0
		B3			1			1			1			1			1
	C		0	0		0	0		0	0		0.75	0		0	0	
	D		1	1		1	1		1	1		1	1		1	1	
		D1			1			1			1			1			1
	F		0	0		0	0		0	0		0	0		0.25	0	
Field	A		1	1		1	1		1	1		1	1				
		A1			0			0.5			0			0			
		A2			1			1			1			1			
		A3			0.5			0.25			0			0.25			
	B		0.5	0		0.5	0		1	0		0.5	0.5				
		B1			0			0			0			0.25			
		B3			0			0			0			0.5			
	D		0.25	0.25		0	0		0.5	0.5		1	0.5				
		D1			0.25			0			0.5			0.5			

[a] Columns with white background show frequencies of multilocus groups A-F detected with *phlD*-T-RFLP or *phlD*-DGGE analysis. Columns with grey background show frequencies of individual DGGE bands detected by *phlD*-DGGE.

phlD amplification. The amplified products were digested with single or combinations of all enzymes and separated with gel electrophoresis. On agarose gels the bands of interest (terminal fragments) showed the expected sizes of about 220 bp for genotype D, 390 bp for genotype C, 530 bp for genotype E, 540 for genotype A, 550 bp for genotype F and 590 for genotype B (data not shown). Furthermore, DNA extracts from spiked soil samples showed the same fragment sizes as samples from overnight cultures. PCR amplification of the *phlD* gene of lysed bacteria using the labelled reverse primer BPR4-HEX and BPR4-FAM showed a less strong signal than the amplification with the unlabelled primer, whereas BPR4-FAM showed a stronger amplification than BPR4-HEX (data not shown). T-RFLP analysis using BPR4-FAM and BPR4-HEX of lysed overnight cultures and DNA extracts from soil spiked with single strains showed peaks with sizes 216 bp for genotype D, 389 bp for genotype C, 524 bp for genotype E, 532 bp for genotype A, 537 bp for genotype F and 582 bp for genotype B. The sizes of the fragments differed by 1 (group D) to 10 bp (group B) from the predicted sizes of the sequence analysis of reference strains. Fragment sizes between runs differed at maximum 1 bp. DNA extracts of rhizosphere samples from 3-week old maize plants spiked with representatives of all groups lacked to resolve the peak of group F when amplified with BPR4-HEX (Fig. 2A), but not with BPR4-FAM (Fig. 2B). All 40 tested reference strains showed the expected peaks when amplified with BPR4-FAM. The detection limit of the T-RFLP approach in spiked rhizosphere samples was 5×10^4 CFU/g for strain *P. fluorescens* CHA0 respectively 5×10^3 CFU/g for strain F113.

3.2. Abundance of total and phlD$^+$ *Pseudomonas spp. on roots of maize and different wheat lines*

The abundance of total and DAPG-producing *Pseudomonas spp.* on the roots of the wheat cultivars Casana, Fiorina, Frisal, and Bobwhite S98 26 as well as the maize hybrid DK315 (only greenhouse) was assessed after six weeks of growth in the greenhouse and in the field. The total cultivable *Pseudomonas* population sizes ranged from log 7.3 (Frisal, greenhouse) to log 8.1 CFU/g root (Casana and Frisal, field, Table 2). Population sizes of *phlD*$^+$ pseudomonads were lower and ranged from log 4.8 for wheat line Fiorina (field) to log 7.3 CFU/g roots for wheat line Bobwhite SH98 26 (greenhouse). Statistical analysis of variance of both experiments together by two-way Anova showed

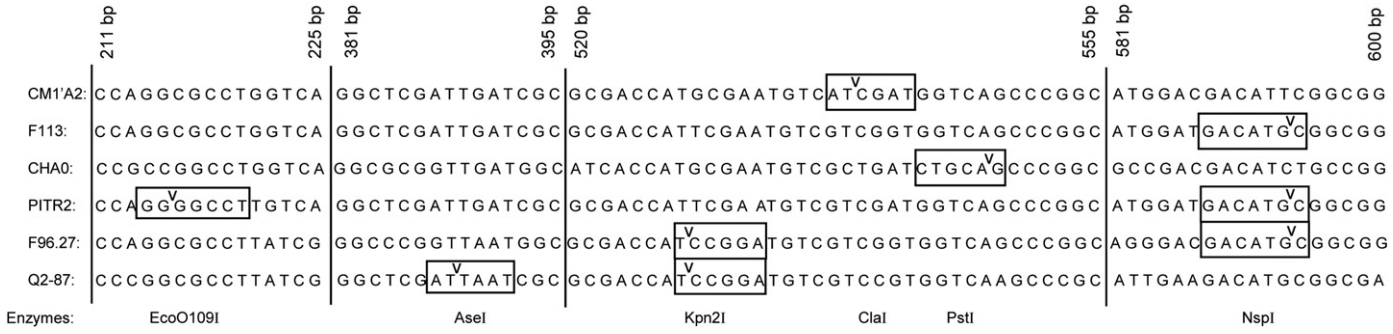

Fig. 1. Alignment of *phlD* sequences of *P. fluorescens* strains representative for the six multilocus groups (A–F) as defined by Frapolli et al., 2007) starting from the labelled reverse primer BPR4. Multilocus group A is represented by strain CM1′A2, B by strain F113, C by strain Q2–87, D by strain PITR2, E by strain F96.27 and F by strain CHA0. Numbers of bp indicate the position within the 629 bp fragment of the *phlD* gene derived from the PCR amplification of reference strains with primers B2BF and BPR4-FAM. ^{V} indicates the cutting positions of the different enzymes.

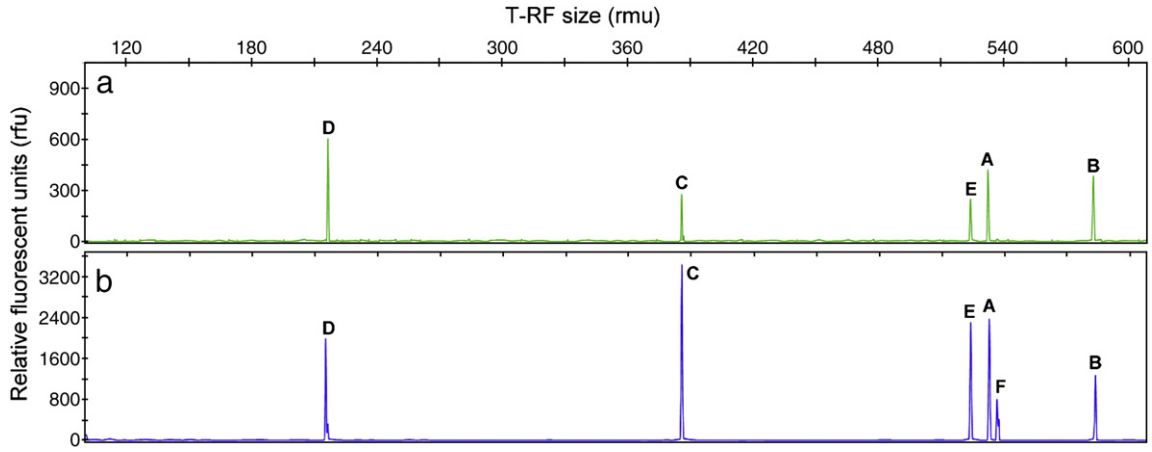

Fig. 2. *PhlD*-T-RFLP profiles of reference strains representing the six multilocus groups in spiked maize rhizosphere samples after the PCR amplification of the *phlD* gene with primers B2BF/BPR4-HEX (a) and B2BF/BPR4-FAM (b). Multilocus groups are represented by *P. fluorescens* strains CM1′A2 (group A), F113 (group B), Q2–87 (group C), PITR2 (group C), F96.27 (group E) and CHA0 (group F). T-RF sizes are represented in relative migration units (rmu) calculated with size standard LIZ600 (Applied Biosystems, Foster City, Ca).

significant differences ($p<0.001$) between field and greenhouse for both, total Pseudomonas and *phlD*$^+$ *Pseudomonas* populations, but no cultivar effect. The *phlD*$^+$ population sizes on the roots of plants grown in the greenhouse were one to two log units higher than those on roots of plants grown in the field (Table 2). When the two experiments were analyzed separately a slight cultivar impact on total pseudomonads ($p=0.05$) and a strong impact on *phlD*$^+$ pseudomonads ($p<0.001$) was observed in the greenhouse experiment, while no cultivar impact was observed in the field experiment. In the greenhouse Fiorina was the cultivar which accumulated most total pseudomonads and Bobwhite the cultivar which accumulated most *phlD*$^+$ pseudomonads. The wheat variety Frisal and the maize cultivar DK315 accumulated the smallest amounts of both total and *phlD*$^+$ pseudomonads.

3.3. T-RFLP and DGGE analysis of phlD$^+$ Pseudomonas spp. on roots of maize and different wheat lines grown in the greenhouse and in the field

The developed *phlD*-T-RFLP approach was used to identify *phlD*$^+$ pseudomonads representing the six multilocus groups defined by Frapolli et al. (2007) in the rhizosphere of four wheat lines and one maize line (only greenhouse) in two experiments (greenhouse and field) with the same soil. The three multilocus groups A, B and D were detected in both experiments, whereas the groups C and F were only detected in the greenhouse experiment (Table 3). Group C was detected in 3 samples of wheat line Bobwhite S98 26 and Group F only in one of the maize line DK315 (Table 3). Comparing the two experiments the genotypes A, B and D were more frequent in the greenhouse experiment than in the field samples (Table 3).

To compare the results of the *phlD*-T-RFLP approach, *phlD*-DGGE analysis was applied to analyze the same samples from the two experiments. *PhlD*-DGGE analysis resulted in a total of seven different bands (Table 3). Sequencing of these bands showed that they share high (99 to 100%) similarity with *Pseudomonas* reference strains belonging to multilocus group A, B and D (Table 4). The sequence of band D1 shows 100% identity with *P. fluorescens* strain C10–186, those of bands A1, A2 and A3 show 99 to 100% identity to *P. fluorescens* P97.30 and sequences of bands B1, B2 and B3 show 99 to 100% identity with *P. fluorescens* strains K93.2, K94.38 and C10–181 (Table 4).

In the greenhouse multilocus groups A, B and D were detected for all cultivars in all four replicates by both methods. In contrast to the T-RFLP approach, however, DGGE analysis did not detect multilocus groups C and F. In the field multilocus groups A and D were detected on the roots of all cultivars by both methods, except on cultivar Fiorina where group D was not detected. Multilocus group B was detected on all cultivars by T-RFLP analysis, by DGGE analysis, however only on Bobwhite.

Cluster analysis of T-RFLP (Fig. 3) and DGGE data (Figs. 3 and 4) showed that greenhouse samples and field samples cluster separately. The separation of field and greenhouse samples was more pronounced when the analysis had been performed with DGGE band frequencies than with multilocus group frequencies.

4. Discussion

In this study we developed a novel *phlD*-T-RFLP approach to discriminate DAPG-producing pseudomonads corresponding to the six phylogenetic multilocus groups determined by Frapolli et al. 2007. The method based on the amplification of the *phlD* gene using primers B2BF and BPR4-FAM followed by a multiple digest with the six enzymes BspI, NspI, AseI, EcoO109I, Kpn2I and PstI allows a fast and reliable discrimination between the six groups from lysed DNA of overnight cultures and of DNA extracted from root and rhizosphere samples of wheat and maize grown in natural soil under greenhouse and field conditions.

Fragment sizes of digested PCR products on agarose gels showed the expected sizes, whereas the sizes of the terminal fragments determined by T-RFLP analysis were lower than expected (by one to ten bp) for almost all multilocus groups. These differences between predicted and observed fragment sizes can be explained due to differences in migration patterns of the fluorescent dyes of our samples (FAM and HEX) and the standard (LIZ). This effect has already been shown in other studies (Hahn et al., 2001, Kaplan and Kitts, 2003, Tu et al., 1998). For example in the study performed by Hahn et al. (2001), it is shown that fluorescein labelled fragments (FAM, 6-FAM, HEX, JOE, NED and TET) migrate differently compared to rhodamine

Table 4
Sequenced *phlD*-DGGE-bands and their correlation to the multilocus groups (Frapolli et al., 2007) based on comparison with reference strains.

DGGE-band	Length of sequence (bp)	Accession number ref. strain	*P. fluorescens* Ref. strain	Sequence identity (%)	*PhlD* cluster (Frapolli et al., 2008)	Multilocus group (Frapolli et al., 2007)
D1	559	EF554333.1	C10–186	100	6	D
A1	548	EF424747.1	P97.30	99	5	A
A2	611	EF424747.1	P97.30	100	5	A
A3	492	EF424747.1	P97.30	100	5	A
B1	553	EF424746.1	K93.2	99	2	B
B2	501	EF424745.1	K94.38	100	2	B
B3	480	EF554332.1	C10–181	99	1	B

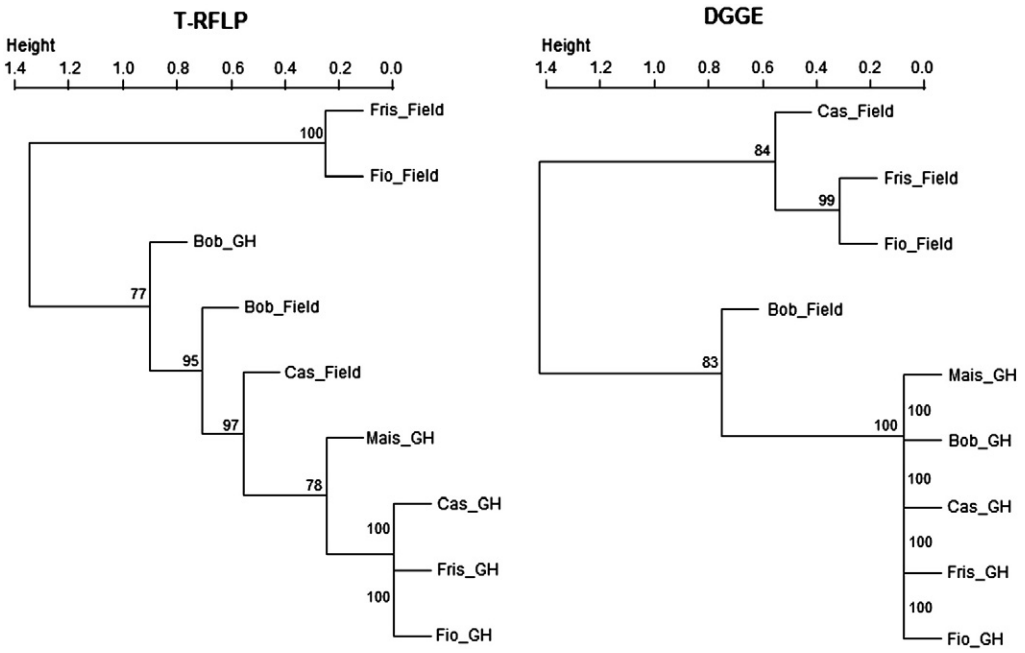

Fig. 3. DGGE and T-RFLP dendrograms based on *phlD*-multilocus profiles obtained from wheat roots grown in the greenhouse (GH) or in the field (Field). The cluster analysis was based on Euclidean distances and constructed with the complete method using R (version 2.12.0).

labelled fragments (ROX, TAMRA). Similar results are also indicated in the study performed by Kaplan and Kitts (2003), in which the drift of the measured T-RF is also mainly explained by different migration patterns of the ROX-labelled standard and the FAM-labelled sample. These authors additionally found that the purine content of the sequence is negatively correlated with the T-RF drift and therefore also has an impact on the migration of the T-RF. The differences in purine content could, in our case, explain the different drifts of the fragments of group A (539–532 bp), group E (528–524 bp) and group F (548–537 bp) which have similar fragment sizes but large differences in T-RF drifts.

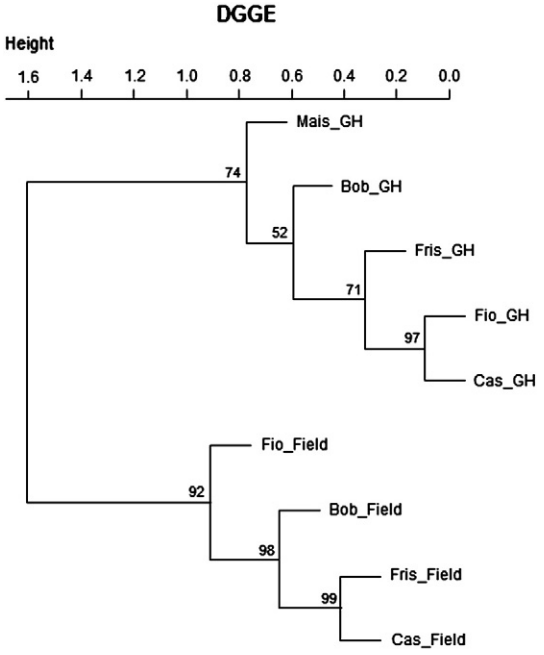

Fig. 4. DGGE dendrogram based on *phlD*-band patterns obtained from wheat roots grown in the greenhouse (GH) or in the field (Field). The cluster analysis was based on Euclidean distances and constructed with the complete method using R (version 2.12.0).

T-RFLP analysis of rhizosphere spiked with reference strains of all multilocus groups failed to detect multilocus group F when samples were amplified with reverse primer BPR4-HEX. Multilocus group F was however detected in the same samples with the same conditions with PBR4-FAM. Therefore we hypothesize, that BPR4-FAM has a better amplification efficiency than BPR4-HEX. A possible reason for this is that HEX is a hexa-chloro derivate of FAM and has six additional chloride atoms rendering it a heavier tag, which could lead to a lower annealing efficiency of the primer. This, in combination with the already low amplification efficiency of group F achieved with the primer pair B2BF/BPR4 most probably leads to a lack of amplification of the *phlD* fragment. Therefore only BPR4-FAM was used for further analysis. The detection limit was determined in spiked rhizosphere samples with strains CHA0 and F113. The two strains were chosen because it was already shown by McSpadden Gardener et al. (2001), that the amplification of the *phlD* gene with primers B2BF and BPR4 is less effective in strains Pf-5 and CHA0 (group F) than in strains Q2–87 (group C) or F113 (group B). Indeed, the detection limit of strain *P. fluorescens* CHA0 (5×10^4 CFU/g rhizosphere) was ten times higher than that for F113 (5×10^3 CFU/g rhizosphere. These detection limits are in an acceptable range because the minimal population level for a PGPR strain to be plant beneficial has to be higher than 10^5 CFU/g root (Haas and Défago, 2005, Weller et al., 2007). Compared to the *phlD*-DGGE method of Frapolli et al. (2008) with a detection limit of 10^7 CFU/g soil for *P. fluorescens* strain CHA0 (group F) our T-RFLP method allows the detection of group F pseudomonads at much lower abundances. The lower detection limit of T-RFLP approach can mainly be explained by the high resolution of the used technology and the improved PCR protocol. However, the PCR protocol of the T-RFLP with a higher cycle number and a longer annealing time could lead to skewed allele representation compared to *phlD*-DGGE. Our results though did not indicate such effects as no changes in allele representation were observed between the amplification of reference strains from pure cultures and from spiked rhizosphere samples (data not shown).

The developed T-RFLP method was applied to identify the different multilocus groups on roots of different wheat cultivars and the maize hybrid DK315 in a greenhouse and also in a field experiment. In both experiments multilocus goups A, B and D were detected whereas groups D and F were only detected in the greenhouse experiment. The

detected multilocus groups were more frequent in the greenhouse experiment than in the field samples, which are in line with the finding that the abundance of *phlD*$^+$ pseudomonads was about one log higher in the greenhouse experiment than in the filed experiment. The fact that group E was not detected in any of the samples is most likely due to the absence of group E pseudomonads in the used soil. However, it also is possible that the population sizes in any of the samples were below the detection limit of the used methods. Furthermore we compared the results of the *phlD*-T-RFLP analysis with *phlD*-DGGE analysis. The *phlD*-DGGE analysis revealed a total of seven bands, representing the multilocus groups A, B and D. Thus both methods detected the same dominant multilocus groups and in the greenhouse experiment also at the same frequencies for all wheat varieties. However, in the field, multilocus groups D and B were less frequently detected with *phlD*-DGGE than with *phlD*-T-RFLP. Additionally multilocus groups C and F were only detected with *phlD*-T-RFLP. Therefore we suggest that our new *phlD*-T-RFLP method can detect lower population sizes of *phlD*$^+$ pseudomonads than the *phlD*-DGGE method described by Frapolli et al. (2008) and provides more complete information on the presence of the six multilocus groups in rhizosphere or soil samples. With *phlD*-DGGE we could not detect all major groups of *phlD*$^+$ pseudomonads detected by *phlD*-T-RFLP. However, the *phlD*-DGGE gave us additional information on subgroups within the detected multilocus groups because different genotypes belonging to the same multilocus group can be distinguished (Fig. 4). The more detailed information obtained by the DGGE analysis also leads to a clear separation of greenhouse and field samples into two clusters (Fig. 4), whereas cluster analysis performed with frequencies of multilocus groups (regardless of the detection method used) show the same tendencies but not such a clear grouping (Fig. 3).

In conclusion, we developed a novel T-RFLP approach for the detection of and discrimination between *phlD*$^+$ biocontrol pseudomonads belonging to six phylogenetically different groups in the rhizosphere. The method proved to be a simple high throughput approach with an improved sensitivity compared to *phlD*-DGGE (Frapolli et al., 2008). Since the six groups of DAPG producers detected by the T-RFLP method most probably correspond to six different species it will be interesting to apply the new method to investigate whether certain soil types or crop species and varieties have preferences for specific phylogenetic groups of *PhlD*$^+$ biocontrol pseudomonads.

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