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Development and evaluation of SCAR markers for a *Pseudomonas brassicacearum* strain used in biological control of snow mould

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ABSTRACT

Biological control microorganisms have long been promoted as an alternative to conventional pesticides. Before registration of a microbial biocontrol product for commercial sale, it must be evaluated as regards potential spread and persistence after release. In this study, strainspecific sequence-characterized amplified region (SCAR) markers were developed to monitor the biocontrol candidate strain *Pseudomonas brassicacearum* MA250, which is effective against snow mould (*Microdochium nivale*). One SCAR marker, OPA2-73, was used in quantitative real-time PCR (Q-PCR) on samples from a climate chamber experiment in which winter wheat seeds were treated with the bacterium or a chemical control agent, or left untreated. The results showed that MA250 persisted for up to 3 weeks after sowing on the kernel residues and also colonized the roots of treated seedlings. Total MA250 cell numbers on biocontrol treated seedlings after three weeks were approximately 10^6 cells, compared with the original inoculum of 10^6–10^7 cells per seed. Corresponding cell numbers of MA250 on chemically treated and untreated seedlings were below the detection limit. This study shows that SCAR marker OPA2-73 is a specific and sensitive tool for monitoring the biocontrol microorganism MA250 in environmental samples.

1. Introduction

Snow mould, also known as pink mould, is a major concern in many countries because the disease causes great crop damage (Parry et al., 1995). The pathogen responsible is the fungus *Microdochium nivale,* previously named *Fusarium nivale* (Samuels and Hallett, 1983). Winters when wet, unfrozen soil is covered by a layer of snow are especially favorable for this disease-causing agent (Schneider and Seaman, 1987; Lipson et al., 1999). Today, most plant pathogens are primarily controlled by conventional chemical fungicides, but much research has been devoted to developing alternative, more environmental-friendly and sustainable biocontrol strategies employing antagonistic microorganisms (Mathre et al., 1999; Harman, 2000; Gerhardson, 2002; Montesinos, 2003). Several isolates belonging to *Pseudomonas* spp. have been demonstrated to have biocontrol potential against a range of plant pathogens (Smiley, 1979; Parke et al., 1991; Friedlender et al., 1993; De la Fuente et al., 2004; Haas and Défago, 2005; Weiss et al., 2007). Some have been further developed into commercial products, e.g. Cedomon® and Cerall® (Lantmännen-BioAgri, Sweden) for controlling stinking smut, leaf or glume blotch and Fusarium wilt (Hökeberg et al., 1997) and BlightBan® (NUFarm Americas Inc, Illinois, US) for controlling fire blight (Johnson and Stockwell, 1998).

Replacing conventional pesticides with biocontrol microorganisms requires an evaluation of potentially harmful side-effects before the microorganisms can be approved and registered as products (Cook et al., 1996; Montesinos, 2003). For example, introduction of new organisms to an ecosystem may cause unwanted non-target effects on resident organisms in the environment in addition to the anticipated biocontrol effect, particularly if the organism is new to the site (van Veen et al., 1997; Andersen and Winding, 2004). Several investigations suggest that such effects are usually transient and will not have long-term effects on the populations (Cook et al., 1996; Mathre et al., 1999; van Elsas and Migheli, 1999; Boland and Brimner, 2004; Winding et al., 2004). Besides safety, monitoring the populations of the introduced organism will give information on the population dynamics of the biocontrol agent, and can therefore be used for optimization of application levels. In order to draw conclusions regarding the probability and extent of the spread and persistence of applied microbial biocontrol agents, a method for monitoring a specific microbial strain in environmental samples is needed. New possibilities to identify DNA sequences unique to the particular strain offer the resolution needed for strain-specific identification (Olive and Bean, 1999). After identification of specific sequences, detection in the environment can be based on direct DNA extraction from samples without prior culturing, followed by PCR with specific primers (Paran and Michelmore, 1993; Abbasi et al., 1999; Olive

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and Bean, 1999). In the present study, we used the typing method random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990; Micheli et al., 1994), to identify strain-specific DNA sequences from a biocontrol bacterium and develop primers that amplify these sequences in DNA from environmental samples. Specific sequence-characterized amplified regions (SCAR) of genomic DNA, differing in as little as one base pair, are enough to distinguish the presence of a particular strain (Abbasi et al., 1999; Hermosa et al., 2001; Pujol et al., 2005). By quantifying these markers, it is possible to monitor the levels and persistence of the applied biocontrol microorganism (Hintz et al., 2001; Dauch et al., 2003; Dodd et al., 2004; Pujol et al., 2004).

In this study, we constructed and evaluated SCAR markers for the strain *Pseudomonas brassicacearum* MA250, which has documented biocontrol effect on snow mould in winter wheat (Levenfors et al., 2007). In order to test the markers with regard to specificity and sensitivity, they were used to monitor MA250 in samples from a climate chamber trial with wheat, where the bacterium was inoculated by seed treatment. The main strengths of utilizing the SCAR method are strain-specific identification of MA250, the possibility to optimize the biocontrol application strategy, and monitoring of the persistence and spread of the strain for safety assessment purposes.

2. Materials and methods

2.1. Strains used in the study

The biocontrol strain used throughout this study for development of SCAR markers was *P. brassicacearum* MA250, 16S-rDNA NCBI Accession No. DQ886486 (Levenfors et al., 2007). The microbe was originally isolated from the roots of an oilseed rape [canola] plant (*Brassica napus*) at a field near Uppsala, Sweden, and belongs to the *P. brassicacearum/P. thivervalensis* sub-group (Achouak et al., 2000). *Pseudomonas brassicacearum* MA250 is available through Lantmännen-BioAgri. A selection of other *P. brassicacearum* strains, other closely related Pseudomonads, and DNA from various bacteria commonly found in agricultural soil were used as reference microorganisms (Tables 1 and 2).

2.2. Identification of potential SCAR marker sequences

2.2.1. RAPD-PCR

All *Pseudomonas* reference strains (Table 1) were cultured in Tryptic Soy Broth (TSB) at 26 °C overnight and genomic DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies). RAPD-PCR was performed using a MiniCycler™ (MJ Research) with defined decamer primer sets (OPA and OPX; Operon Biotechnologies). These two sets have been used more extensively than other primer sets in successful amplification (Catara et al., 2000; Castrillo et al., 2002; Dodd and Stewart, 2003; Sazakli et al., 2005). The PCR amplification protocol (modification of De Clercq et al., 2003) was a step-down consisting of: initial denaturation at 95 °C for 3 min, annealing at 38 °C for 2 min and extension at 72 °C at 2 min, followed by 95 °C for 40 s, 37 °C for 45 s, and 72 °C for 90 s. The annealing temperature was then decreased every cycle by one degree down to 33 °C. The last cycle of 95 °C for 40 s, 33 °C for 45 s, and 72 °C for 90 s was repeated 39 times followed by 72 °C for 10 min. Reagent conditions were: 10 mM Tris–HCl pH 9.0, 3.0 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP, 1 U cloned Taq polymerase (GE Healthcare) and 0.8 μM RAPD primer (De Clercq et al., 2003), and 10 ng of genomic DNA were used in each reaction of 25 μL. PCR products were separated on a 1.6% agarose gel in 0.5× TBE buffer. Bands of interest were excised, gel-purified (QIAGEN Gel Extraction Kit), cloned into a PCR II vector (Invitrogen), and sequenced with M13 forward (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) universal primers for the vector on an ABI PRISM® 3700 DNA Analyzer. To check whether the two SCAR markers contained known sequences, e.g. encoding a specific protein, we blasted the obtained sequences against DNA and protein databases (www.ncbi.nlm.nih.gov/blast/blast.cgi).

2.2.2. Development of primers for SCAR-PCR

The sequences of the cloned markers were used as templates to design specific SCAR-PCR primers. The DNA stretches corresponding to the RAPD primers were elongated to 25 bp (Table 3) and the new synthesized primers were tested against the reference strains and species (Tables 1 and 2), both with pure DNA and in autoclaved soil inoculated with bacteria (10^9 cells to 10 g of soil, original wet weight, incubated overnight at room temperature).

Table 1
Pseudomonas reference strains for RAPD-PCR and specificity tests of SCAR primers. The majority of the strains were obtained from culture collections.

Strain	Culture collection/reference
Pseudomonas aeruginosa	LMG 1242
Pseudomonas chlororaphis	LMG 5832
Pseudomonas corrugata	LMG 2172
Pseudomonas marginalis pv. *marginalis*	LMG 1243
Pseudomonas brassicacearum	CFBP 6505
Pseudomonas brassicacearum	CFBP 6510
Pseudomonas brassicacearum	CFBP 6511
Pseudomonas brassicacearum	CFBP 6517
Pseudomonas brassicacearum	CFBP 6518
Pseudomonas brassicacearum	DSM 13227
Pseudomonas thivervalensis	DSM13194
Pseudomonas kilonensis	DSM13647
Pseudomonas fluorescens, SBW25	Thompson et al. (1995)
Pseudomonas brassicacearum, 520-1	Sikorski et al. (2001)
Pseudomonas brassicacearum, MA250	Levenfors et al. (2007)

Table 2
Additional reference strains representing soil bacteria for evaluation of SCAR marker specificity. The material was obtained in the form of pure DNA.

Strain	Culture collection/reference
Pseudomonas aeruginosa	CCUG 241
Pseudomonas denitrificans	CCUG 1783
Pseudomonas denitrificans	CCUG 2519
Pseudomonas fluorescens	ATCC 33512
Pseudomonas mendocina	ATCC 25411
Pseudomonas putida	CCUG 2479
Pseudomonas stutzeri	CCUG 29240
Pseudomonas stutzeri	ATCC 11607
Pseudomonas stutzeri	ATCC 17588
Pseudomonas stutzeri JM 300	DSM 10701
Pseudomonas stutzeri	ATCC 14405
Paracoccus denitrificans	ATCC 19367
Paracoccus denitrificans, Pd 1222	NCCB 97099
Paracoccus denitrificans	CCUG 13798
Paracoccus denitrificans	CCUG 30144
Alcaligenes sp.	DSM 30128
Achromobacter denitrificans	CCUG 407T
Alcaligenes eutrophus	ATCC 17699
Alcaligenes faecalis	ATCC 8750
Alcaligenes faecalis	ATCC 19018
Blastobacter denitrificans	ATCC 43295
Comamonas testosteroni	CCUG 1426T
Hyphomicrobium denitrificans	DSM 1869T
Cupriavidus metallidurans	CCUG 13724
Arthrobacter chlorophenolicus	DSM 12829T
Pseudomonas fluorescens Mi32	Hallin and Lindgren (1999)
Rhizobium meliloti, RM	Throbäck et al. (2004)
Bradyrhizobium japonicum, B.jap	Throbäck et al. (2004)
Nitrosospira sp., III7	Utåker and Nes (1998)
Nitrosospira sp., III2	Utåker and Nes (1998)
Nitrosospira sp,. AF	Utåker et al. (1995)
Nitrosospira sp., 40KI	Utåker et al. (1995)
Nitrosospira sp., A4	Aaakra et al. (1999)