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STRAIN-SPECIFIC SCAR MARKERS FOR THE DETECTION OF *TRICHODERMA HARZIANUM* AS12-2, A BIOLOGICAL CONTROL AGENT AGAINST *RHIZOCTONIA SOLANI*, THE CAUSAL AGENT OF RICE SHEATH BLIGHT

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In order to identify a specific marker for *T. harzianum* AS12-2, a strain capable of controlling rice sheath blight caused by *Rhizoctonia solani*, UP-PCR was performed using five universal primers (UP) both separately and in pairwise combinations. The application of two UP primers resulted in the amplification of unique fragments from the genomic DNA of *T. harzianum* AS12-2, clearly distinguishing it from other *Trichoderma* strains. The unique fragments had no significant sequence homology with any other known sequence available in databases. Based on the sequences of the unique fragments, 14 oligonucleotide primers were designed. Two primer sets amplified a fragment of expected size from the DNA of strain *T. harzianum* AS12-2 but not from any other examined strains belonging to *T. harzianum*, to other *Trichoderma* species assayed, or to other common fungi present in paddy fields of Mazandaran province, Iran. In conclusion, SCAR (sequence characterized amplified regions) markers were successfully identified and rapid, reliable tools were provided for the detection of an effective biocontrol *Trichoderma* strain, which can facilitate studies of its population dynamics and establishment after release into the natural environment.

Keywords: Rhizoctonia solani – Trichoderma harzianum – biological control – rice sheath blight – SCAR markers

INTRODUCTION

The application of fungal biocontrol agents (BCAs) is becoming an increasingly important alternative to chemicals in crop protection against weeds, insects and fungal pathogens in both agriculture and forestry [3]. In order to predictably and successfully apply BCAs to combat plant diseases in the field, their biology and ecology should be well understood [10]. The commercial use of filamentous fungi from the genus *Trichoderma* as BCAs must be also preceded by monitoring the fate and behavior of the released Trichoderma strain and its population dynamics in soil [1, 20], as the registration of a specific BCA as a pesticide requires a risk assessment focused on

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its persistence and multiplication in the environment. These legal requirements emphasize the use of monitoring methods that can accurately identify the released strain, distinguish it from the native microbial community and track its population dynamics over time [21]. Ecological studies in the rhizosphere and soils were performed using *Trichoderma* strains transformed with reporter genes encoding β-glucuronidase and green fluorescent protein [4]. Although these methods are suitable for detection and monitoring of *Trichoderma* strains under natural conditions, this would involve the release of genetically modified organisms (GMOs) into the environment [20]. Due to strong public opposition to the release of GMOs, the application of endogenous markers, e.g. unique DNA sequences that distinguish a single strain from all others, is a more preferred option. Nucleic acid-based genetic markers can be highly specific, and high sensitivity has been achieved with PCR-based assays in the identification and detection of many species and strains of fungi [16].

Universally primed PCR (UP-PCR) is a useful tool for the characterization and grouping of isolates in order to study their genetic relatedness [6, 12]. UP primers primarily target intergenic, more variable areas of the genome and for this reason, the method is especially suitable for detection of intraspecific variation [6]. The UP-PCR technique has been applied to *Trichoderma* strains in previous studies [6, 8, 12–15] and has proven useful for strain and species identification [13]. Furthermore, UP-PCR generated, strain-specific fragments have the potential to be converted into sequence characterized amplified regions (SCARs) [19]. SCARs allow the differentiation of the wild type biocontrol strain from the autochthonous population of fungi belonging to the same species or genus. Strategies based on strain specific SCAR markers were developed for potential BCAs within the genus *Trichoderma*, both in conventional and real-time PCR [1, 7, 8, 20].

The aim of the present study was to identify specific, endogenous molecular markers that allow the detection of *T. harzianum* Rifai AS12-2, an excellent biocontrol strain against the rice sheath blight pathogen *Rhizoctonia solani* AG1-1A [17, 18], thereby providing tools for the monitoring of survival, spread and population dynamics of this strain in rice fields.

MATERIAL AND METHODS

Fungal cultures

All *Trichoderma* strains applied in this study were isolated from paddy rice fields of Mazandaran Province located in the south coasts of Caspian Sea, Iran (Table 1) [17, 18]. They belong to six species, i.e. *T. harzianum* Rifai, *T. virens* (Miller, Giddens & Foster) von Arx, *T. atroviride* P. Karst, *T. hamatum* (Bonord.) Bainier, *T. asperellum* Samuels, Lieckf. & Nirenberg and *T. brevicompactum* Kraus, Kubicek & Gams. All strains were maintained on potato dextrose agar (PDA) (Merck, Germany) slants at 4 °C. The biocontrol strain *T. harzianum* AS12-2 was selected for evaluation of the possibility of its detection with SCAR markers. Rice field isolates of *Rhizoctonia*

solani J. G. Kühn, *Gibberella fujikuroi* (Sawada) Wollenw., *Magnaporthe grisea* (T. T. Hebert) M. E. Barr, *M. salvinii* (Catt.) R. A. Krause & R. K. Webster, *Bipolaris oryzae* (Breda de Haan) Shoemaker, *Nigrospora oryzae* (Berk. & Broome) Petch, *Aspergillus* sp. and *Penicillium* sp. applied in this study are listed in Table 1.

DNA extraction

For DNA isolation, mycelial plugs were placed on sterile cellophane disks covering the surface of yeast extract agar (5 g yeast extract, 5 g dextrose and 20 g agar per litre) plates and incubated for 2–3 days at room temperature. Fresh mycelia were scraped

Table 1
Fungal strains used during this study

Species	Isolate code[a]	GenBank accession number of ITS sequences
T. harzianum	AD1-1[b], AS3-3, AS3-5, AS4-1, AS4-2, AS4-3, AS5, AS15-3, AS19-1, AS19-2, AS19-3, AS19-4, AS20-1, AS20-2, AS20-3, AS20-4, AS20-5, AS21-1, AS21-2, AS21-3, AS22-1, AS22-2, AS22-3, AS22-4, BS7-1, BS7-2, BS7-3, SS1-1, SS1-2, SS10-4, SS10-5, DS201, DS202, DS203, DS204, DS205, DS322, BP4, BL1-2, BL1-3, BL1-4, BL2-1, BL2-5, BL3-2, BL3-3, BL3-5, BL3-9, BL4-1, BL6-3, BL7-3, BL7-5, BL8-2, BL9-2, BL9-3, BL9-5, BL9-6, BLP2, BLP7, BLP8	EU821780[c]
	AS2-1, AS2-2, AS2-3, AS17-2, BLP5, BS1-1, BS1-3, BS1-4, SS10-2	EU821781
	AD7, AS15-1, AS15-4, AS15-6, AS15-7, AS16-1, AS17-3, AS17-6	EU821782
	CS1-1, CS4-2, DS603, DS604, DS801, DS803	EU821783
	AD1-2, AD6	EU821784
	DS301	EU821785
	SS6-1	EU821786
	SS7-1	EU821787
	SS10-3	EU821788
	AS12-2	EU821789
	SS8-4	EU821790
	AS16-3, AS16-4, AS16-5, BL2-2, BL2-3, BL3-7, BL3-8, BL7-4, BL7-6, BL8-5, BLP1, CS4-3, DS403, DS404, DS502, DS507, DS555	EU821791
	SS6-2, SS6-21, SS103	EU821792
	DS303, DS304	EU821793

Table 1 (cont.)

Species	Isolate code[a]	GenBank accession number of ITS sequences
T. virens	AD1-3, AD1-5, AS1-1, AS1-2, AS3-1, AS3-2, AS3-4, AS6-1, AS6-2, AS6-3, AS6-4, AS10-1, AS10-2, AS10-3, AS10-4, AS10-5, AS10-6, AS10-7, AS11-1, AS11-2, AS11-3, AS11-4, AS14-1, AS14-2, AS14-3, BL1-5, BL3-1, BL8-1, BS1-2, BS2-1, BS2-2, BS2-3, BS2-4, BS2-5, BS2-6, BS2-7, CS1-2, CS1-3, SS109, DS402, DS404, DS405, DS421, DS601	EU821794
	AS15-2, AS15-5, AS16-2, AS16-22, AS18-1, AS18-2, AS18-3, AS18-4, BS3-1, BS3-2, BS3-3, BS3-4, BS3-5, BS3-6, BS3-11, SS7-3, DS100, DS508, DS509, DS901, DS905	EU821795
	AS17-1, AS17-4, AS17-5, SS8-1	EU821796
T. atroviride	AS8, AS18-5, CS2-1, CS5-1, DS111, DS112, DS121, DS122, DS501, DS602, DS606	EU821797
T. hamatum	SS11-2, SS108-2, DS302	EU821798
T. asperellum	BS3-8	EU821799
T. brevicompactum	DS701	EU821800
Rhizoctonia solani	RBL1	HM211085
Gibberella fujikuroi	MF79	N/A[d]
Magnaporthe grisea	VK33	N/A
M. salvinii	VK28	N/A
Bipolaris oryzae	MF14	N/A
Nigrospora oryzae	MF12	N/A
Aspergillus sp.	MF44	N/A
Penicillium sp.	MF53	N/A

[a]All isolates obtained from rice fields in Mazandaran province, Iran.
[b]Isolates were grouped according to their ITS genotypes.
[c]Identical GenBank accession numbers indicate that the ITS sequences of the corresponding isolates are identical.
[d]N/A = not available.

off and ground in liquid nitrogen with a pestle in a mortar. Total DNA was extracted with the GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA) according to the manufacturer's instructions.

UP-PCR fingerprinting

To identify a specific marker for *T. harzianum* AS12-2, a pilot study was carried out using five universal primers (Table 2) separately or in pair-wise combinations. UP-PCR was performed as described by Lübeck et al. [15] using 40 ng of primers (20 ng for each of the UP primers when used pairwise) and 40–60 ng template DNA in a final volume of 20 µl. The annealing temperature was optimized using a gradient from 52 °C to 68 °C. Each amplification included a negative control without template DNA. The amplification conditions using the MJ Mini™ Gradient Thermal Cycler (Bio-Rad, USA) were as follows: 30 cycles with DNA denaturation at 92 °C for 50 s (first denaturation step at 94 °C for 3 min), primer annealing at 52–56 °C for 70 s, and primer extension at 72 °C for 60 s; and a final extension step at 72 °C for 3 min.

UP-PCR amplification products were subjected to electrophoresis at 100 V for 1 h in 1.7% agarose gel prepared in 1 × TBE (Tris-borate-EDTA; pH 8.0) buffer containing ethidium bromide. GeneRuler™ 100-bp DNA ladder Plus (Fermentas Life Sciences) was used as the molecular weight marker. DNA was made visible with the BioDoc-It System (UVP, USA) and photographed with a UP-895CE video graphic printer (Sony, Japan).

UP primers were screened with purified genomic DNA of 26 selected isolates of *Trichoderma harzianum* (corresponding to different ITS genotypes, including *T. harzianum* AS12-2) and *T. virens*, the two most common species in rice fields of Mazandaran province.

Cloning and sequencing of UP-PCR amplicons

UP-PCR fragments specific to *T. harzianum* AS12-2 were purified from the agarose gel with the GenElute Minus EtBr Spin Column kit (Sigma, USA) and ligated into the pCR®2.1-TOPO® vector from TOPO TA Cloning® Kit (Invitrogen, USA) according to the instructions of the manufacturer. Chemically competent *Escherichia coli* TOP10F´ OneShot® cells were transformed with ligated DNA, plated on LB medium containing kanamycin (35 µg/ml), X-gal (40 µg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside) (100 mM). Recombinant plasmid DNA was extracted using

Table 2
UP primers applied in the study

Name	Length (mer)	Sequence (5′ to 3′)	References
L45	17	GTAAAACGACGGCCAGT	Bulat et al. [5, 6]
3-2	16	TAAGGGCGGTGCCAGT	Bulat et al. [5]
AA2M2	16	CTGCGACCCAGAGCGG	Lübeck et al. [11]
AS15inv	17	CATTGCTGGCGAATCGG	Bulat et al. [5]
L15/AS19	15	GAGGGTGGCGGCTAG	Lübeck et al. [12]

Mini-M Plasmid DNA Extraction System (VIOGENE, USA) and sequenced on both strands with M13 forward and reverse universal primers at Macrogen Inc., South Korea. Homologies to known sequences were searched in the GenBank database using the Basic Alignment Search Tool (BLAST) available online from the National Center for Biotechnology Information [2]. The nucleotide sequences of the cloned UP-PCR fragments were deposited in the GenBank database.

Design of SCAR primers

Specific primers were designed based on visual inspection of both ends and inner part of the sequences of unique UP-PCR fragments and were synthesized at Biocenter Co., Hungary. BLAST searches were performed with the designed primers to ensure that the 3' end of the primers do not show complete homology with any of the known sequences of *Trichoderma*, *R. solani*, other filamentous fungi or the genome of the rice plant.

Diagnostic PCR

The SCAR primer pairs were evaluated by conventional PCR for their ability to specifically amplify a unique product from strain *T. harzianum* AS12-2. The specificity of the SCAR primers was tested using fungal DNA from 198 *Trichoderma* isolates belonging to six species as well as from other fungal species which are common in rice fields in Mazandaran (Table 1). Initially, the genomic DNA of selected *Trichoderma* isolates (belonging to *T. harzianum* and *T. virens*) were screened in PCR reactions covering all possible forward/reverse primer combinations to determine if a unique product was produced for *T. harzianum* AS12-2. The PCR reaction conditions were the same as those described above for UP-PCR. PCR optimization was performed by testing several annealing temperatures from 64 °C to 68 °C using a temperature gradient. The amplified products were resolved electrophoretically in a 1% agarose gel. pUC Mix Marker, 8 (Fermentas Life Sciences) was used as a molecular weight marker.

RESULTS

UP-PCR analysis of Trichoderma *strains*

All five UP primers were found to generate fingerprints with a relatively high number of bands. In nearly all cases the fingerprints were highly variable among the *T. harzianum* strains tested, whereas the strains belonging to other species proved to be less diverse, i.e. the fingerprints were quite similar although not identical, indicating a relative homogeneity for these species (results not shown).

PCR optimization was performed by testing several annealing temperatures from 52 °C to 56 °C using a temperature gradient. The optimal temperature was 55.7 °C.

T. harzianum AS12-2 was distinguished from all other *Trichoderma* isolates by generating amplification products of unique size with the single primers AS15inv and AA2M2 (Fig. 1), as well as their pair-wise combination. Three distinct amplicons (UF-AS15inv, UF-AA2M2 and UF-AS15inv/AA2M2) were generated reproducibly from the DNA of strain AS12-2 and were absent in the other examined isolates of *T. harzianum* and *T. virens*. The reproducibility of these amplicons was confirmed by repeating UP-PCR analysis of DNA samples derived from independent extractions. The amplicons UF-AS15inv and UF-AA2M2 clearly distinguished *T. harzianum*

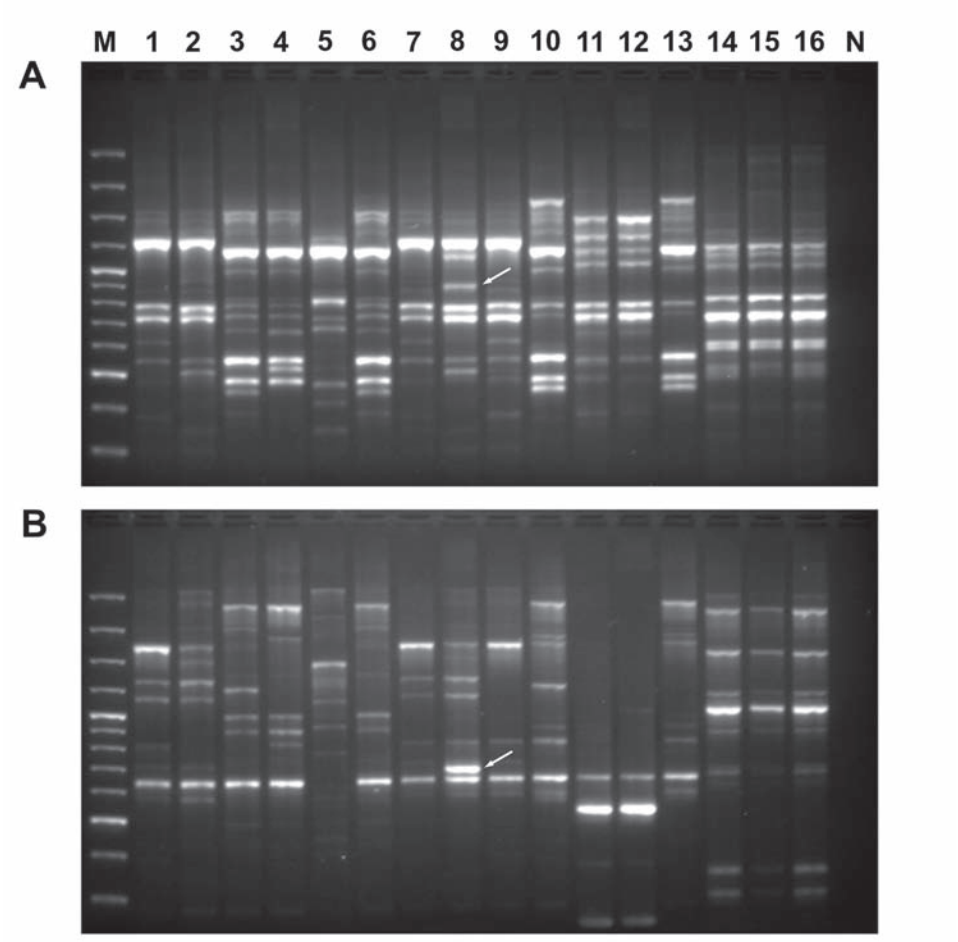

Fig. 1. UP-PCR banding profiles of selected isolates generated with UP primer AS15inv (A) and AA2M2 (B). Lanes 1–13: *T. harzianum* strains AS20-2, AS2-1, AD7, DS801, DS301, SS7-1, SS10-3, AS12-2, AS4-1, AS16-3, SS6-2, DS304 and BL2-2, respectively. Lanes 14–16: *T. virens* strains AD1-3, AS16-22 and AS10-6, respectively. The unique amplicons to *T. harzianum* AS12-2 are indicated by arrows. Lane M: GeneRuler 100-bp DNA ladder. Lane N: negative control without template DNA

AS12-2 from other *T. harzianum* and *T. virens* isolates (Fig. 1). UF-AS15inv/AA2M2 was also diagnostic for *T. harzianum* AS12-2, however, some *T. harzianum* strains had fragments of similar size (data not shown). The approximate sizes of the amplicons UF-AS15inv, UF-AA2M2 and UF-AS15inv/AA2M2 were 900, 650, and 850 bp, respectively.

Sequence analysis of the diagnostic amplicons and design of strain-specific primers

Sequencing of the unique fragments, i.e. UF-AS15inv, UF-AA2M2 and UF-AS15inv/AA2M2 from *T. harzianum* AS12-2 revealed DNA sequences of 905, 684 and 842-bp in length, respectively, which were deposited in the GenBank database under accession numbers GQ201447, GQ201448 and GQ201449. These fragments showed no significant similarity to any other known sequences deposited in the GenBank database, further supporting their specificity. The sequences of the three DNA fragments were distinct, with no sequence homology with each other.

Table 3
Specific primers designed from sequences of unique UP-PCR fragments

Name	Length (mer)	Sequence (5′ to 3′)	T_m (°C)
Forward primers			
15invFL1	22	CTGTGCTCCAATTGATCGACGA	57.21
15invF2	22	TTGCTGGCGAATCGGAGGATAC	58.58
15invFS1	23	GCTTCAGTCGTATCAACCTTGGT	56.93
AA2M2FL1	22	GCCGGAGACTTACCTGAACCAT	58.17
AA2M2FS1	24	ACCATCATCGGGTCGTTATTAAGC	57.00
M2invFL1	21	CCGAACATTGCACGCAGTTCT	58.41
M2invF2	22	CGGTAAGAACCGAACATTGCAC	58.93
M2invFS1	25	GTCAAATAGCCACTTGGACATGTCA	57.39
Reverse primers			
15invRL1	26	GACAACTTGAAGGTAGACGAATCGTC	57.67
15invRS1	22	GTGTGATGTGCAAATCGGCAAG	57.50
AA2M2RL1	24	AACTCGCGAGGCAACTTTATTCAG	57.91
AA2M2RS1	23	CGTGCTGTCTAAAGTCTACGACC	57.13
M2invRL1	22	GCACCACTATGGGCCTCTAACT	57.89
M2invRS1	22	GCCGTCAAATTACACACGCATC	57.33

In order to develop a rapid, simple, and specific diagnostic PCR assay for *T. harzianum* AS12-2, the UP-PCR markers were converted into SCAR markers. Totally, 14 oligonucleotide primers including eight forward and six reverse primers were designed (Table 3), and the GenBank database was screened for sequences with the greatest degree of homology to the primers. Very close sequences were not found.

Diagnostic PCR

SCAR primers were tested in all possible combinations with the DNA of selected strains of *T. harzianum* and *T. virens*. The optimal annealing temperature was found to be 67 °C. Only two primer pairs were able to strongly amplify single, unique bands from *T. harzianum* AS12-2. Primer combinations 15invFL1/15invRL1 and 15invF2/15invRL1 (Fig. 2) designed from the sequence of the fragment UF-AS15inv amplified the predicted, approximately 796-bp and 627-bp DNA fragments, respectively, when the genomic DNA of *T. harzianum* AS12-2 was used as template. The specificity of these two primer pairs for *T. harzianum* AS12-2 was evaluated more extensively in amplifications with genomic DNA templates of all *Trichoderma* strains listed in Table 1 as well as with the DNA of other fungal genera, including various common inhabitants of rice fields. No products were generated with any genomic DNA tested, except from the DNA of *T. harzianum* AS12-2 (data not shown).

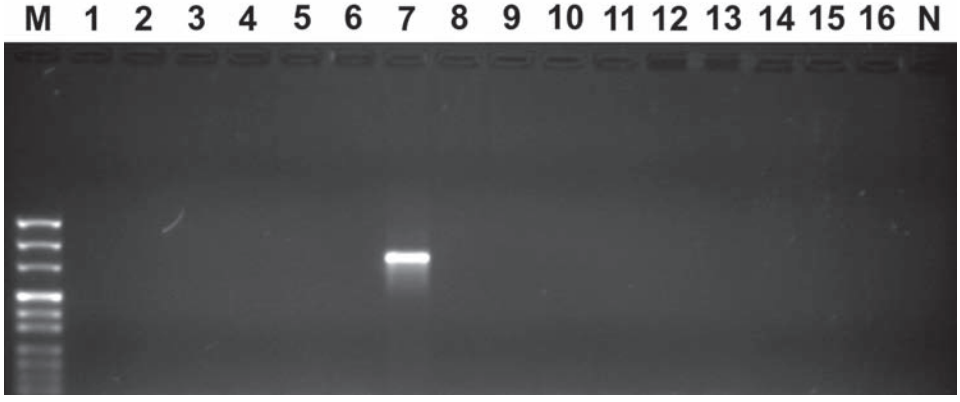

Fig. 2. SCAR marker diagnostic for *T. harzianum* AS12-2. Shown are the PCR products amplified with strain-specific primers 15invF2/15invRL1. Lanes 1–13: *T. harzianum* strains AS20-2, AS2-1, AD7, DS801, DS301, SS7-1, AS12-2, SS10-3, AS4-1, AS16-3, SS6-2, DS304 and BL2-2, respectively. Lanes 14–16: *T. virens* strains AD1-3, AS16-22 and AS10-6, respectively. Lane M: pUC Mix Marker, 8. Lane N: negative control without template DNA

DISCUSSION

The identification of particular strains of fungi on the basis of their DNA requires the characterization of discriminating DNA targets. It is especially important in the case of *Trichoderma* strains widely used in the biological control of soil-borne plant-pathogens. There is a need for the development of molecular approaches that are faster than the time-consuming and labor-intensive traditional microbiological methods, thereby well suited to analyze large numbers of samples and facilitate the monitoring of *Trichoderma* strains as BCAs in natural environments.

Zimand et al. [22] used the RAPD procedure for the specific identification of isolate T-39 in the *T. harzianum* group. They found that a set of nine primers was necessary to distinguish T-39 from other isolates of the group. Abbasi et al. [1] stated that three RAPD markers were needed to distinguish *T. hamatum* 382 from other *T. hamatum* isolates. UP-PCR fingerprinting is another simple and reliable technique that can be used to detect genetic differences even at the strain level. It has proven to be a valuable tool for studying DNA polymorphisms in *Trichoderma* [6, 8, 12–15]. By the application of this approach we have identified three UP-PCR markers enabling the detection of a specific *T. harzianum* strain, which is an effective BCA against the rice sheath blight pathogen *R. solani* [17]. The identified markers discriminate strain AS12-2 from other strains of *T. harzianum* as well as from other *Trichoderma* species. The results indicate the applicability of the UP-PCR method for distinguishing closely related strains and for studies of diversity and genetic structure of populations.

Although we successfully applied UP-PCR analysis to detect differences between individual *Trichoderma* strains, this technique may not be suitable for the development of a sensitive, rapid, and reliable monitoring system for *T. harzianum* AS12-2 in rice fields, therefore the identified UP-PCR markers were converted into SCAR markers.

Hermosa et al. [9] developed a RAPD fragment-based, strain specific SCAR marker to distinguish *T. atroviride* 11, a BCA against soil-borne fungal plant pathogens from 42 other *Trichoderma* strains (including BCAs) belonging to 13 species. Although the probability with which a SCAR marker can be lost during field trials is considered relatively low, the authors suggested that it can be further reduced by the development of more strain specific markers for a single strain. In the recent study, two SCAR markers specific to *T. harzianum* AS12-2 have been developed. Each of the primer pairs 15invFL1/15invRL1 and 15invF2/15invRL1 amplified a unique product from the DNA of *T. harzianum* AS12-2. No bands were detected in the reactions containing DNA from the other fungi examined, demonstrating that these specific primers do not cross-react with other strains of *T. harzianum*, other *Trichoderma* species and other fungi commonly occurring in paddy fields. The procedure was consistent and the results were reproducible: DNA extraction and PCR amplification procedures were repeated and the same results were obtained. With these two specific primer pairs, strain *T. harzianum* AS12-2 could be distinguished from 112 other *T. harzianum* strains, 70 strains of *T. virens* as well as 11, 3, 1 and 1 strains belonging to *T. atroviride*, *T. hamatum*, *T. asperellum* and *T. brevicompactum*, respectively, indicating that we have identified two molecular markers that are suitable for the specific detection of this BCA. These SCAR markers have the potential to be applied for the monitoring of *T. harzianum* AS12-2 after its release into rice fields; furthermore, they can also facilitate biomass quantification in the case of this BCA and provide information about its population dynamics and establishment in the soil or plant materials.

In conclusion, the results of this study indicate that the application of UP-PCR and subsequent design of SCAR primers based on sequence data from unique fragments

proved to be an efficient strategy for the development of strain-specific detection methods in the case of biocontrol *Trichoderma* strains.

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