

# Identification and evaluation of two diagnostic markers linked to *Fusarium* wilt resistance (race 4) in banana (*Musa* spp.)

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**Abstract** *Fusarium* wilt caused by the fungus *Fusarium oxysporum* f. sp. *cubense* race 4 (FOC4) results in vascular tissue damage and ultimately death of banana (*Musa* spp.) plants. Somaclonal variants of in vitro micropropagated banana can hamper success in propagation of genotypes resistant to FOC4. Early identification of FOC4 resistance in micropropagated banana plantlets is difficult, however. In this study, we identified sequence-characterized amplified region (SCAR) markers of banana associated with resistance to FOC4. Using pooled DNA from resistant or susceptible genotypes and 500 arbitrary 10-mer oligonucleotide primers, 24 random amplified polymorphic DNA (RAPD) products were identified. Two of these RAPD markers were successfully converted to SCAR markers, called ScaU1001 (GenBank accession number HQ613949) and ScaS0901 (GenBank accession number HQ613950). ScaS0901 and ScaU1001 could be amplified in FOC4-resistant banana genotypes (“Williams 8818-1” and Goldfinger), but not in five tested banana cultivars susceptible to

FOC4. The two SCAR markers were then used to identify a somaclonal variant of the genotype “Williams 8818-1”, which lost resistance to FOC4. Hence, the identified SCAR markers can be applied for a rapid quality control of FOC4-resistant banana plantlets immediately after the in vitro micropropagation stage. Furthermore, ScaU1001 and ScaS0901 will facilitate marker-assisted selection of new banana cultivars resistant to FOC4.

**Keywords** Banana (*Musa* spp.) · RAPD · SCAR markers · *Fusarium* wilt · *Fusarium oxysporum* f. sp. *cubense* (race 4)

## Abbreviations

FOC *Fusarium oxysporum* f. sp. *cubense*  
FOC4 *Fusarium oxysporum* f. sp. *cubense* race 4  
RAPD Random amplified polymorphic DNA  
SCAR Sequence-characterized amplified region  
p.i. Post inoculation

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## Introduction

Banana and plantain (*Musa* spp.) are important crop plants in tropical and sub-tropical zones. Annual banana production in the world is estimated at about 106 million tons [1]. Most of the currently cultivated bananas are triploid, highly sterile and are multiplied by vegetative propagation [2]. Due to the lack of classical breeding techniques, the genetic diversity of banana is relatively low. Banana production is adversely affected by a various pests and diseases, particularly *Fusarium* wilt disease, which is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (FOC). [3]. *Fusarium* wilt, also known as Panama

disease, has been first reported in Panama in the end of the nineteenth century. Fusarium wilt is one of the most lethal and destructive banana diseases throughout the world [3–5]. Fusarium wilt is a classic vascular wilt disease, during which the fungus enters into xylem vessels of the host plant. Fungal proliferation in the xylem causes leaf wilting and ultimately death of the whole plant [6]. Based on disease symptoms of different banana cultivars, FOC has been classified into four physiological races [7, 8]. *Fusarium oxysporum* f. sp. *cubense* race 4 (FOC4) is the most virulent FOC race and possesses a relatively broad host range. Most banana cultivars, producing more than 80% of the world's harvest (including the important Cavendish subgroup) are susceptible to FOC4 [9].

Although extensive studies have been carried out during the last two decades [10], most chemical and mechanical measures against FOC are inefficient. Currently practiced application procedures with fungicides such as carbendazim are time-consuming and costly [11, 12]. Biological control of Fusarium wilt by inoculation of antagonistic fungi and bacteria isolated from suppressive soils has been considered as an alternative approach to chemical control [13–16]. Additional measures, such as application of fertilizers and cost-effective soil amendments to change the soil pH, may also reduce survival of FOC [17, 18]. However, these techniques only control FOC to a certain extent. In general, it has been proved to be extremely difficult to control Fusarium wilt, especially when induced by FOC4 [19]. Therefore, identification of new banana cultivars resistant to FOC4 remains the most promising option to solve the economically important Fusarium wilt problem [20].

Various studies demonstrated that random amplification of polymorphic DNA (RAPD) markers and their conversion into sequence-characterized amplified region (SCAR) markers are beneficial and robust tools for identification and detection of genetic loci associated with FOC resistance. SCAR markers linked to FOC resistance genes have been developed for melon [21], cotton [22], ginger [23], chickpea [24], tomato [25], and eggplant [26]. Furthermore, PCR methods with specific primers and DNA isolated from FOC have been used for identification and characterization of different physiological FOC races [27, 28]. In banana however, SCAR markers linked to genetic loci linked to Fusarium wilt resistance have not been identified yet.

In this study, we identified RAPD markers associated with FOC4 resistance and converted two of them successfully into SCAR markers. The obtained SCAR markers will help to diagnose FOC resistance of micro-propagated banana during the in vitro propagation stage and facilitate molecular marker-assisted selection of novel cultivars resistant to FOC4.

## Materials and methods

### Plant material

Seven banana genotypes known to be either resistant or susceptible to FOC4 were used in this study. These genotypes had diverse genetic constitution and ploidy levels and were obtained from the Banana Field Germplasm Bank of the Southern Subtropical Crop Research Institute of CATAS (Guangdong, China). The genotypes susceptible to FOC4 were cv. Williams 8818 (AAA genomic group), cv. Grand Naine (AAA group), cv. Gros Michel (AAA group), cv. Brazilian (AAA group), and cv. Tianbao (AAA group). The genotypes resistant to FOC4 were cv. Goldfinger (AAAB group) and a mutant derivative of cv. Williams 8818, named “Williams 8818-1”, which was obtained from an ethyl methanesulphonate mutagenesis screen. The “Williams 8818-1” phenotypically differs from the parent genotype cv. Williams with respect to the density and size of banana fingers. It is therefore likely that the “Williams 8818-1” represents a somaclonal variant of cv. Williams.

All banana plantlets used in this study were multiplied by an in vitro micropropagation procedure [29]. Plantlets were cultured on a regeneration medium containing macro and micro mineral salts supplemented with vitamins of the Murashige–Skoog medium [30]. Micropropagated plants were first grown in a culture room at  $25 \pm 2^\circ\text{C}$ . Rooted plantlets were transferred to pots filled with sterile soil. For acclimatization, plants were maintained in a humid and shady environment for 12–15 days. Plants were then transferred to a greenhouse and kept for 8 weeks.

### DNA extraction and RAPD analysis

Genomic DNA from young leaves was extracted with the cetyltrimethylammonium bromide method [31] with some minor modifications. Random amplified polymorphic DNA (RAPD) analysis was performed with 20 ng of genomic DNA as template in a reaction volume of 25  $\mu\text{l}$  containing 0.2 mM dNTP, 2.0 mM  $\text{MgCl}_2$ , 2.5  $\mu\text{l}$  10  $\times$  Taq polymerase buffer, 1 unit Taq DNA polymerase (Takara, Japan) and 0.1  $\mu\text{M}$  of each RAPD primer. In total, 500 random 10-mer primers (Operon Technologies, Alameda, CA and Roth, Karlsruhe, Germany) were tested. PCR samples were covered with 2  $\mu\text{l}$  of mineral oil and amplification reactions carried out using a MJ research thermocycler (model PTC-225; MJ Research). The PCR conditions were as follows: (i) a pre-denaturation step for 5 min at  $94^\circ\text{C}$ , (ii) a synthesis step with 40 cycles ( $94^\circ\text{C}$  for 40 s,  $38^\circ\text{C}$  for 60 s and  $72^\circ\text{C}$  for 90 s) and (iii) a final extension step of 10 min at  $72^\circ\text{C}$ . PCR products were then subjected to electrophoresis on 1.2 or 1.5% agarose gels in TBE buffer (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA). DNA was

visualized under UV light after staining with  $0.5 \mu\text{g ml}^{-1}$  ethidium bromide.

#### Cloning and sequencing of PCR products

PCR products were excised from 1.5% agarose gels and DNA was purified using a DNA purification kit (Takara, Japan). The purified DNA was then cloned into the pGEM-T easy vector (Promega, Southampton, UK) according to the manufacturer's instructions. Plasmids were then transformed into *Escherichia coli* DH5 $\alpha$  and DNA was isolated with a plasmid extraction kit (Takara, Japan). Sequencing was carried out by the Takara Biotech Company. Sequence comparisons with databases were performed with the Blastn program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

#### PCR with SCAR primers

Based on each cloned RAPD product, two 20–25-mer oligonucleotides were designed as SCAR primers. In total, 24 primer pairs were synthesized by extending the original 10-mer RAPD primer with the following sequenced 10–14 nucleotides at the 3' end [32]. Amplification of genomic DNA was performed in a 25  $\mu\text{l}$  reaction mixture containing 20 ng of template DNA, 2 mM  $\text{MgCl}_2$ , 0.2  $\mu\text{M}$  of each specific SCAR primer, 200  $\mu\text{M}$  dNTP, 2.5  $\mu\text{l}$  10  $\times$  Taq polymerase buffer and 1 unit Taq DNA polymerase (Takara, Japan). After denaturation at 94°C for 5 min, 35 cycles (94°C for 30 s, different annealing temperatures for 60 s and 72°C for 90 s) were performed followed by a final extension step at 72°C for 10 min. PCR products were separated by electrophoresis and visualized as described above.

Where indicated, a multiplex PCR with additional primers deduced from the *M. acuminata* 18S rRNA gene (GeneBank accession number U42083.1) was performed, resulting in an additional control PCR product (491 bp). Forward and reverse primer sequences were 5'-CATCACA GGATTTTCGGTCTCT-3' and 5'-AGACAAATCGCTCCAC CAAC-3', respectively.

#### Southern blot analysis

Genomic DNA was digested either with *EcoRI*, *HindIII* or *XhoI*, and electrophoretically separated on 1.2% agarose gel. The DNA was then transferred to a nylon membrane (GeneScreen Hybridisation Transfer Membrane, PerkinElmer Life Sciences, Inc., Boston, MA, USA). Digoxigenin-labeled probes were prepared by using a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) and purified DNA of the identified SCAR markers. After hybridization at 65°C for 18 h, membranes were washed twice with  $0.1 \times \text{SSC}$  (15 mM NaCl,

1.5 mM Na-citrate, pH 7.0) containing 0.1% sodium dodecyl sulphate at 65°C for 20 min. Detection of DNA was performed according to the supplier's instructions.

#### Inoculation experiment

FOC4 was obtained from the Laboratory of Tropical and Subtropical Fruit (South China Agricultural University, Guangzhou, China). Spores were harvested after prolonged incubation of cultures on half strength potato dextrose agar plates at room temperature. Formation of spores was counted at weekly intervals for a period of 1 month. For inoculum preparation, a single-spore was added to a Nash-PCNB plate [33] and the culture was kept at 25°C for 10 days. The fungal culture was then centrifuged at 5000 rpm for 5 min and the pellet was re-suspended in double distilled water. The optical density of the fungal suspension was adjusted to  $10^6$  per mm with sterile water.

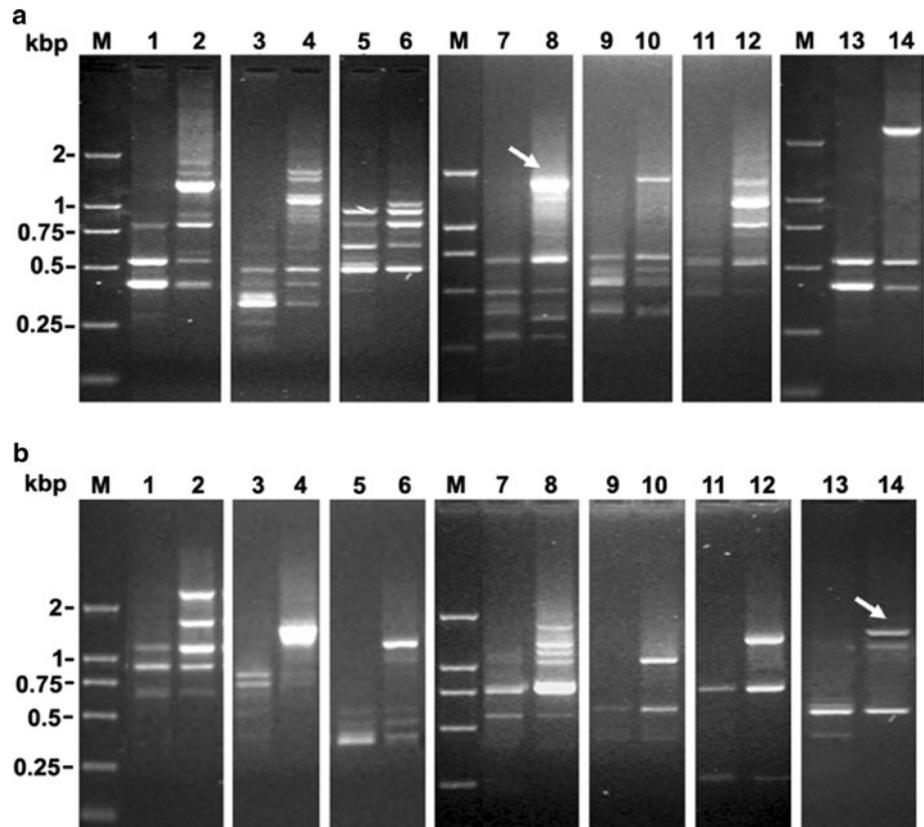
Young banana plants (genotype “Williams 8818-1”), grown in pots under greenhouse conditions at  $26 \pm 2^\circ\text{C}$  for 8 weeks, were inoculated with the prepared FOC4 suspension. Roots were wounded with a scalpel and then dipped into 15 ml of fungal inoculum suspension in a Petri dish (9 cm in diameter) for 30 min. Mock inoculation of injured control plants was performed with double distilled water. In total, 40 plants (including micropropagated plants of the identified FOC4-inoculated somaclonal variant “plant No. 6”) were used. After inoculation, plants were kept under field conditions. Field trials were conducted at the China Southern Subtropical Crop Research Institute of CATAS in Zhanjiang (Guangdong Province). Plants were periodically (up to 76 days) assessed for FOC4 disease symptoms (leaf chlorosis, necrosis and complete wilting of plants).

## Results

#### Identification of RAPD markers associated with FOC4 resistance

Genomic DNA of two 7 different banana genotypes either susceptible (Williams 8818, Grand Naine, Gros Michel, Brazilian and Tianbao) or resistant (Goldfinger, “Williams 8818-1”) to FOC4 was isolated from leaves of young plantlets. DNA was then combined to form a pool for the five susceptible genotypes and another pool for the two resistant genotypes. Both DNA pools were then used for identification of RAPD markers using a total of 500 RAPD arbitrary 10-mer primers. In total, 423 primers yielded 2101 bands ranging from 250 to 2500 bp. The number of RAPD bands generated per primer varied from 2 to 8 with an average of five bands per primer. Although the majority

**Fig. 1** Identification of RAPD markers associated with FOC4 resistance. RAPD was performed with a total of 500 arbitrary RAPD primers and pooled DNA from banana genotypes either susceptible (S) or resistant (R) to FOC4. Bands marked with an arrow were successfully converted to SCAR markers. Molecular weight DNA markers are indicated on the left. **a** PCR products of RAPD with primers OPM09 (lane 1 R; lane 2 S), OPO02 (lane 3 R; lane 4 S), OPK08 (lane 5 R; lane 6 S), OPU10 (lane 7 R; lane 8 S), OPU14 (lane 9 R; lane 10 S), OPU19 (lane 11 R; lane 12 S) and OPS01 (lane 13 R; lane 14 S). **b** PCR products of RAPD with primers OPJ12 (lane 1 R; lane 2 S), OPP01 (lane 3 R; lane 4 S), OPP08 (lane 5 R; lane 6 S), OPH15 (lane 7 R; lane 8 S), OPJ4 (lane 9 R; lane 10 S), OPJ11 (lane 11 R; lane 12 S), and OPS09 (lane 13 R; lane 14 S)



of the bands were identical, the use of 14 arbitrary primers (OPH15, OPJ04, OPJ11, OPJ12, OPK08, OPM09, OPO02, OPP01, OPP08, OPS01, OPS09, OPU10, OPU14, and OPU19) resulted in reproducible RAPD products that differed for the two pools. In total, 24 RAPD products were specifically amplified with the DNA pool from the two banana genotypes resistant to FOC4 (Fig. 1).

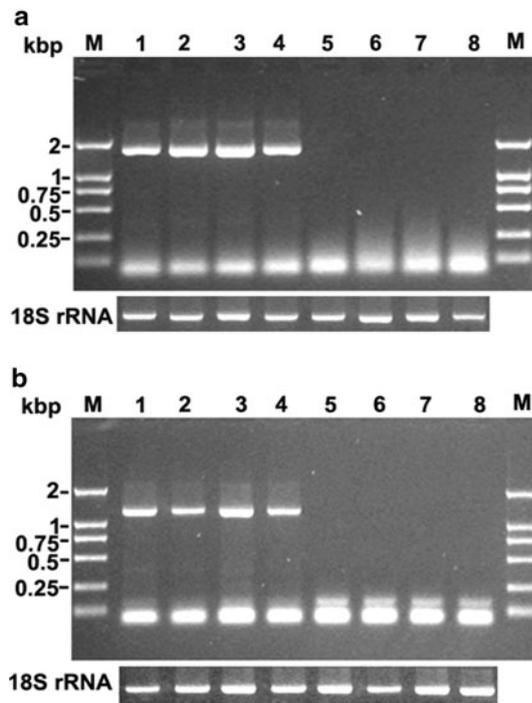
#### Development of SCAR markers associated with FOC4 resistance

The 24 specific RAPD products were cloned into the pGEM-T vector and fully sequenced. As SCAR markers are more reliable and locus-specific than RAPD markers [34], longer SCAR primers were designed based on the original RAPD 10-mer primer sequence and the sequenced amplification product. The use of two SCAR primer pairs and template DNA from banana genotypes resistant to FOC4 (Goldfinger and “Williams 8818-1”) resulted in amplification of PCR products, whereas no corresponding bands were seen with four other genotypes susceptible to FOC4 (Figs. 2, 3). These two SCAR markers, named ScaU1001 and ScaS0901, had a length of 1694 and 1429 bp, respectively (Table 1). These values corresponded to the size the cloned RAPD sequences. The sequences were deposited at the GenBank database

(accession numbers HQ613949 and HQ613950). Database comparisons (Blastn search against the nucleotide and EST databases at the NCBI homepage) revealed that ScaU1001 and ScaS0901 had no similarities with coding sequences of structural genes. In contrast to ScaU1001 and ScaS0901, reactions with other SCAR primer combinations resulted in several or no bands with template DNA from all tested genotypes, although attempts were made to optimize the PCR conditions [35].

We also characterized the sensitivity of the PCR with the SCAR primers. ScaU1001 and ScaS0901 could be amplified when 10–50 ng template DNA was used in a 25  $\mu$ l reaction mixture. The SCAR primers could be successfully used at a concentration as low as 0.1  $\mu$ M. Under the tested PCR conditions, no non-specific PCR products were detected. Furthermore, the SCAR-PCR system for ScaU1001 and ScaS0901 was improved by adding primers that amplify a 491-bp DNA fragment of the 18S rRNA gene. Such internal control was particularly useful for reactions with DNA from banana genotypes susceptible to FOC4 (Fig. 2).

Southern-blot analysis was performed to elucidate whether ScaU1001 and ScaS0901 correspond to a multi-copy sequence family or to a single locus. DNA from the “Williams 8818-1” digested either with *EcoRI-HindIII* or *EcoRI-XhoI* was used for the Southern-blot analysis.

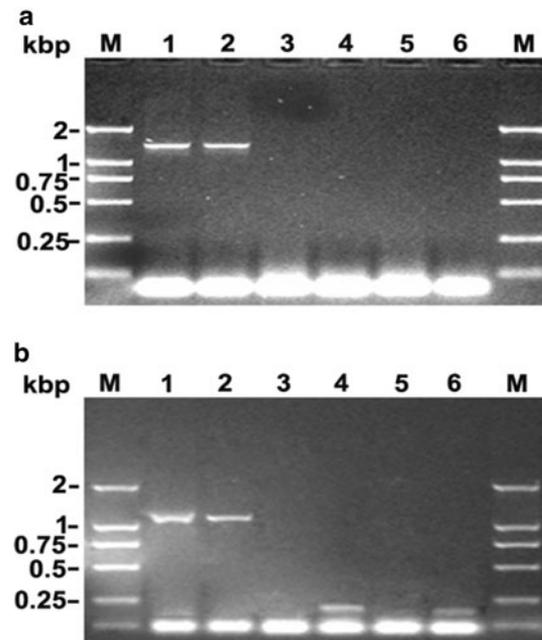


**Fig. 2** Multiplex PCR with SCAR primer sets in combination with 18S rRNA primers to amplify a 491-bp DNA fragment of the 18S rRNA gene. Amplification reactions were performed with genomic DNA isolated from leaves of FOC4-resistant “Williams 8818-1” or FOC4-susceptible “Williams” plants. Molecular weight DNA markers (M) were loaded on both sites of the gel. **a** Multiplex PCR analysis of ScaU1001. Lane 1 pooled DNA from “Williams 8818-1” plants, lane 2–4 DNA from individual “Williams 8818-1” plants, lane 5 pooled DNA from “Williams” plants, lane 6–8 DNA from individual “Williams” plants. **b** Multiplex PCR analysis of ScaS0901 with DNA as indicated in **a**

As shown in Fig. 4, single bands were seen on the Southern blots, indicating that ScaU1001 and ScaS0901 represent single-copy loci.

The identified SCAR markers can be used to diagnose FOC4 resistance in micropropagated plantlets

Plantlets of the FOC4-resistant “Williams 8818-1” micropropagated *in vitro* by a tissue culture procedure were tested for the presence of absence of the SCAR markers ScaU1001 and ScaS0901. DNA was isolated from leaves of 40 randomly selected individual plants cultured under controlled conditions in a growth chamber and PCR reactions were performed with the identified SCAR primer pairs (Fig. 5). Expected DNA bands were seen for 39 plants. However, PCR with DNA of one plant (No. 6 in Fig. 5) did not result in the expected PCR product, i.e., ScaU1001 and ScaS0901 DNA could not be amplified. Hence, plant No. 6 represented a somaclonal variant, which was apparently mutated in the region of ScaU1001 and ScaS0901.



**Fig. 3** Analysis of the SCAR markers ScaU1001 and ScaS0901 in different banana cultivars either resistant or susceptible to FOC4. Amplification reactions were performed with the corresponding SCAR primers and genomic DNA was isolated from leaves. Molecular weight DNA markers (M) are indicated on the left. **a** Analysis of ScaU1001 with genotypes resistant to FOC4 (lane 1 “Williams 8818-1”; lane 2 Goldfinger) and susceptible to FOC4 (lane 3 Williams; lane 4 Grand Naine; lane 5 Gros Michel; lane 6 Brazilian). **b** Analysis of ScaS0901 with genotypes resistant to FOC4 (lane 1 “Williams 8818-1”; lane 2 Goldfinger) and susceptible to FOC4 (lane 3 Tianbao; lane 4 Grand Naine; lane 5 Gros Michel; lane 6 Brazilian)

We further performed an inoculation experiment to test whether the lack of ScaU1001 and ScaS0901 in plant No. 6 is associated with susceptibility to FOC4. “Williams 8818-1” and micropropagated plants of the somaclonal variant (plant No. 6) were first cultivated in sterile soil under greenhouse conditions. After fungal inoculation, plants were kept under field conditions and monitored for disease symptoms (Fig. 6). As expected, FOC4-inoculated “Williams 8818-1” plants were resistant to FOC4. Leaves remained dark-green and the plants did not show any detectable disease symptoms during the whole experimental period. In contrast to these FOC4-resistant plants, the FOC4-inoculated somaclonal variant (plant No. 6) showed first disease symptoms 33 days post inoculation (p.i.). Leaves turned yellow and the symptoms progressed later to younger leaves. At 76 days p.i., most leaves wilted and collapsed. Furthermore, harvested pseudostems analyzed 33 and 76 days p.i. showed necrotic zones, which are typical FOC disease symptoms (Fig. 6). These findings indicate that micropropagated plants from the identified somaclonal variant (plant No. 6) were susceptible to FOC4.

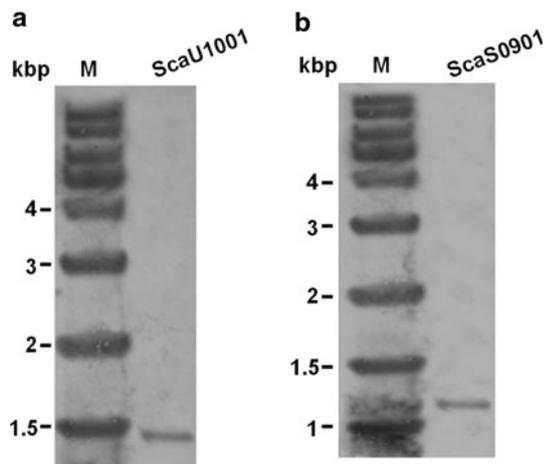
**Table 1** FOC4-associated SCAR markers ScaU1001 and ScaS0901 identified in this study

SCAR	Primer name	Primer sequence (5' to 3') <sup>a</sup>	Product length (bp) <sup>b</sup>	T <sub>m</sub> <sup>c</sup>
ScaU1001	OPU1001forward	<u>ACCTCGGCACT</u> CGAAGACACAT	1694	55
	OPU1001reverse	<u>ACCTCGGCACT</u> ATTACCCATCAT		
ScaS0901	OPS0901forward	<u>TCCTGGTCCCAGT</u> ACAAATAC	1429	54
	OPS0901reverse	<u>TCCTGGTCCC</u> TCTGAATTTTC		

<sup>a</sup> Underlined nucleotides represent the sequences of the original RAPD primers

<sup>b</sup> GenBank accession numbers HQ613949 and HQ613950, respectively

<sup>c</sup> Annealing temperature (°C)



**Fig. 4** Southern blot analysis of SCAR markers ScaU1001 and ScaS0901. Genomic DNA of the FOC-resistant genotype “Williams 8818-1” was digested with the indicated restriction endonucleases. Digoxigenin-labeled DNA of SCAR markers served as probes. Molecular weight DNA markers (M) are indicated on the left. **a** Genomic DNA digested with *EcoRI-HindIII* and hybridized with ScaU1001 DNA as a probe. **b** Genomic DNA digested with *EcoRI-XhoI* and hybridized with ScaS0901 DNA as a probe

## Discussion

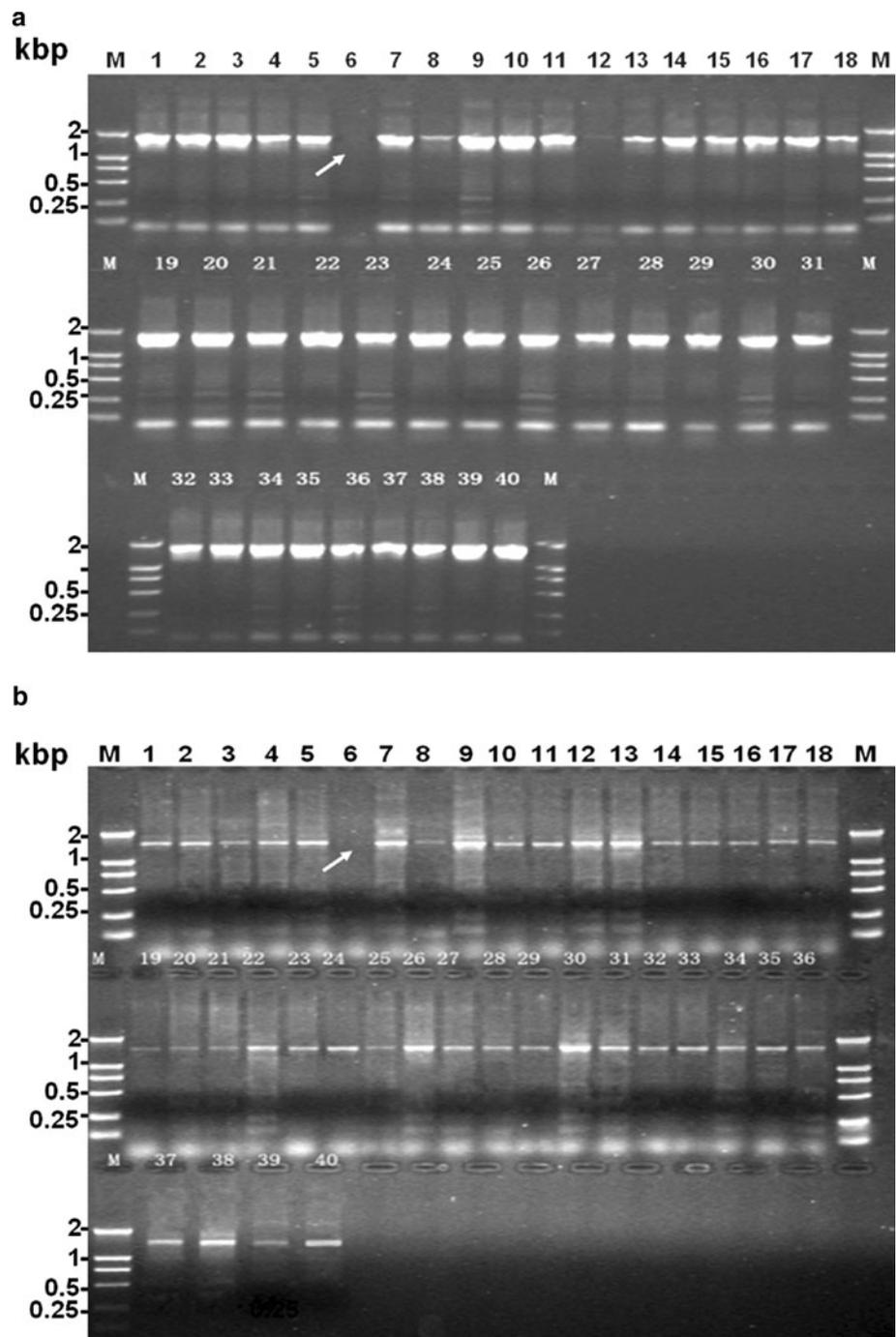
In this study, we developed RAPD and SCAR markers associated with FOC4 resistance. By using the DNA from various banana genotypes either resistant or susceptible to FOC4, reactions with 14 RAPD primers resulted in 24 differentially amplified PCR products. However, the use of RAPD primers often results in amplification of multiple gene loci and the amplification reactions frequently depend on certain PCR conditions [32, 35]. To overcome these disadvantages, we converted the RAPD marker into SCAR markers, which usually give more specific and reproducible results. SCAR markers have been successfully used in research and breeding programs to characterize or identify resistance genes in many plants such as tomato [25, 36], durum wheat [37], melon [21], and sorghum [38]. In our study, two markers, ScaU1001 and ScaS0901, were amplified as single DNA fragments with 20–50 ng

template DNA from FOC4-resistant genotypes (Goldfinger and “Williams 8818-1”), whereas no bands were seen with DNA of five banana genotypes susceptible to FOC4. PCR results obtained with other SCAR primers, however, did not correlate with FOC4 resistance. Future attempts to optimize the SCAR primers and PCR conditions [35, 39] will eventually lead to identification of additional SCAR markers associated with FOC4 resistance.

Analysis of the SCAR markers ScaU1001 and ScaS0901 in a multiplex PCR with an extra pair of primers to amplify an 18S rRNA gene fragment [35, 40, 41] can be considered as novel method to predict FOC4 resistance of young micropropagated banana plants without performing any infection experiment. Using this approach, somaclonal variants lacking ScaU1001 and ScaS0901, such as plant No. 6 in Fig. 5, can be rapidly identified and eliminated at an early stage of the *in vitro* micropropagation procedure. Currently, scientific tests for FOC4 resistance greatly depend on adequate inoculation and disease evaluation methods. Although recently improved [42], phenotypic evaluation of FOC4 resistance in micropropagated banana plantlets remains a difficult and time-consuming process [13, 18], particularly when tested at a large scale. Furthermore, selected plants used for infection experiments are contaminated with the inoculum and cannot be further used. Hence, monitoring micropropagated banana plantlets with the identified SCAR markers ScaU1001 and ScaS0901 is an efficient, safe and rapid alternative method, which allows routine and cost-effective quality control of FOC4-resistant banana plantlets immediately after the micropropagation procedure.

Identification of SCAR markers linked to disease resistance gene loci will also help banana breeders to develop new FOC4-resistant cultivars based on marker assisted selection. SCAR markers are particularly important for banana breeding programs, as spontaneous occurrence of somaclonal variants can hamper success in propagation of selected clones. Indeed, analysis of ScaU1001 and ScaS0901 in 40 micropropagated plantlets of the FOC4-resistant “Williams 8818-1” resulted in the identification of a somaclonal variant, which lacked ScaU1001 as well as

**Fig. 5** Analysis of the SCAR markers ScaU1001 and ScaS0901 in micropropagated plantlets of the genotype “Williams 8818-1”. Leaf DNA was extracted from individual plantlets. Molecular weight DNA markers were loaded on both sites of each gel. The lacking PCR products of somaclonal variant “plant No. 6” are marked with *arrows*. **a** Analysis of ScaU1001 with DNA of 40 individual plantlets (*lanes 1–40*). **b** Analysis of ScaS0901 with DNA of 40 individual plantlets (*lanes 1–40*)

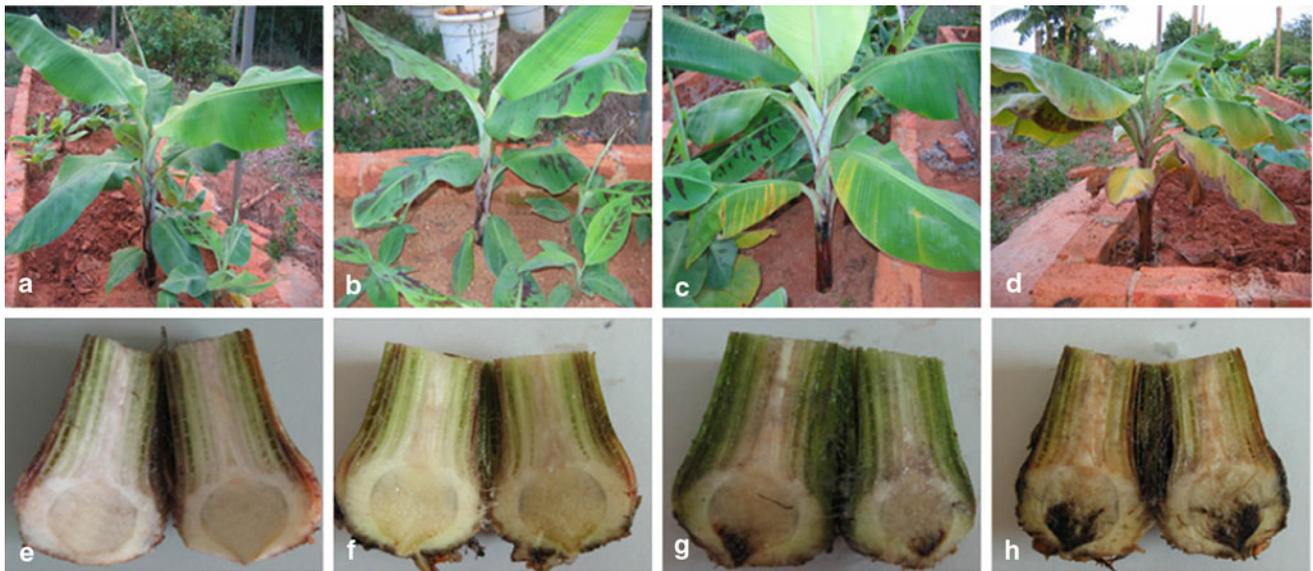


ScaS0901 (plant No. 6 in Fig. 5). A subsequent fungal inoculation experiment with further micropropagated plants showed that this somaclonal variant indeed lost resistance to FOC4, providing additional evidence that ScaU1001 and ScaS0901 are linked to FOC4 resistance. As the identified FOC4-susceptible somaclonal variant lacked both SCAR markers, a single mutation event that simultaneously affected both SCAR markers can be proposed. We hypothesize that ScaU1001 and ScaS0901 are closely linked with a single resistance gene locus. Future work is

required to identify this resistance gene locus by linkage mapping and chromosome walking techniques [22, 24, 43].

### Conclusion

Our results demonstrate that ScaU1001 and ScaS0901 are specific and reliable markers to diagnose FOC4 resistance in individual banana plantlets after in vitro micropropagation. The established multiplex PCR allows rapid



**Fig. 6** FOC4 inoculation experiment with micropropagated plants of the FOC4-resistant genotype “Williams 8818-1” and the identified somaclonal variant “plant No. 6” susceptible to FOC4. **a** FOC4-resistant “Williams 8818-1” plant inoculated with FOC4 33 days p.i. **b** FOC4-resistant “Williams 8818-1” plant inoculated with FOC4 76 days p.i. **c** Disease symptoms of somaclonal variant “plant No. 6” inoculated with FOC4 33 days p.i. **d** Disease symptoms of

somaclonal variant “plant No. 6” inoculated with FOC4 76 days p.i. **e** Pseudostem of FOC4-resistant “Williams 8818-1” plant inoculated with FOC4 33 days p.i. **f** Pseudostem of FOC4-resistant “Williams 8818-1” plant inoculated with FOC4 76 days p.i. **g** Necrotic pseudostem of somaclonal variant “plant No. 6” inoculated with FOC4 33 days p.i. **h** Necrotic pseudostem of somaclonal variant “plant No. 6” inoculated with FOC4 76 days p.i.

identification and cost-effective elimination of somaclonal variants susceptible to FOC4, which thwart large-scale propagation of FOC4-resistant banana cultivars. The SCAR markers will help to rapidly propagate FOC4-resistant genotypes at low costs and also facilitate development of new banana cultivars resistant to FOC4. Finally, the SCAR markers reported in this study form the basis for identification of a linked resistance gene locus in FOC4-resistant banana.

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