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Discrimination of tobacco cultivars using SCAR and RAPD markers

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Abstract: Tobacco genetic purity is crucial to maintain the quality of cigarette products in the tobacco industry. To reduce the difficulties in the discrimination of large number of tobacco cultivars in production practice, we developed a two-step identification strategy by using SCAR and RAPD markers. A total of 53 tobacco cultivars were examined in the study. Initially, all the selected cultivars were divided into four groups, each group consisted of seven to seventeen tobacco cultivars based on difference in phenotypes identified by the SCAR markers S4 and S8. Later, in each group, each tobacco cultivar was identified using RAPD fingerprinting by using one to four polymorphic primers, which were selected from 200 random primers. The results showed that all 53 tobacco cultivars could be effectively distinguished by using only two SCAR and seven RAPD markers. The two-step fingerprinting strategy could be used as a convenient and cost-effective tool to discriminate large numbers of tobacco cultivars for production planning in the tobacco industry.

Keywords: cultivar; fingerprinting; identification; RAPD; SCAR; *Nicotiana tabacum*

Tobacco is one of the most important cash crops of China, where hundreds of cultivars are cultivated in large areas each year (Sun et al. 2016). For a specific cigarette product, usually more than ten tobacco cultivars, which differ significantly in their chemical composition, are used in the blending formula. Therefore, it is vitally important to authenticate the genetic purity of the tobacco cultivar to maintain the consistency of the cigarette's quality (Ma et al. 2012).

In the past 20 years, several DNA fingerprinting technologies such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), sequence-characterized amplified region (SCAR), and simple sequence repeat (SSR) were developed to discriminate tobacco cultivars or their accessions. Among all the molecular markers, RAPD is the most inexpensive and simple marker involved in DNA fingerprinting technologies (Dar et al. 2019). RAPD has been effectively used to analyse the genetic diversity of many tobacco cultivars (Xu et al. 1998; Wang et al. 2003; Yang et al. 2006; Raju 2011).

The object of the current study was to develop an inexpensive and simple method to discriminate the genetic differences of a large number (more than 50) of tobacco cultivars. This study was focused on determining a relatively small number of primers to distinguish these 53 tobacco cultivars, rather than doing a cluster analysis of tobacco cultivars with primers. The procedure consists of developing reliable SCAR markers, selecting reproducible RAPD primers and performing a discrimination analysis of the genetic difference of the tobacco cultivars. More specifically, first, it was necessary to systematically divide the tobacco cultivars into identical groups to develop effective SCAR markers. Then, we used selected reproducible RAPD primers to identify each cultivar in each group. It was expected that the results of this study could lead to the reduced primer usage and improve the discrimination efficiency.

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Fifty-three commonly cultivated local tobacco cultivars were used in the study (Table S1 in the Electronic Supplementary Material (ESM)). For each cultivar, leaf tissues collected from the seedlings (7–9 leaf stage) were used to extract the genomic DNA. The optical density (OD) 260/280 and concentration of the genomic DNA of the 53 tobacco cultivars varied between 1.72–1.96 and 93.3–1234.2 ng/μL, respectively. The RAPD-PCR reactions were performed in a thermal cycler under the following conditions: preheating at 94 °C for 6 min, then 40 cycles of 94 °C for 1 min, 36 °C for 30 s and 72 °C for 90 s, followed by 72 °C for 10 min. The PCR (polymerase chain reaction) products were resolved on 2% agarose gel electrophoresis and visualised by ethidium bromide (EB) staining. The polymorphic RAPD bands, which were present in some of the cultivars, but absent in other cultivars, were purified and ligated with a pUCm-T vector and then transformed into *Escherichia coli* DH5α. The positive clones selected by blue and white spot screening were sequenced by Sangon Biotech in China.

Eight SCAR primers were designed based on the sequences of eight cloned RAPD fragments (Table 1). The SCAR-PCR reactions were performed in a thermal cycler under the following conditions: preheating at 94 °C for 5 min, then 31 cycles of 94 °C for 1 min, 50–65 °C and 72 °C for 50 s, followed by 72 °C for 10 min. The PCR results of the SCAR marker S4-F/S4-R showed that the estimated 525 bp band was present in 24 cultivars, but absent in other 29 cultivars (Figure 1A). While the 374 bp band amplified by the SCAR marker S8-F/S8-R was present in 30 cultivars, but absent in other 23 cultivars (Figure 1B). The other SCAR markers were present in all the tobacco cultivars. Based on the present (marked 1), or absent (marked 0) of the S4 and S8 markers, 53 tobacco cultivars were divided into group I, group II, group III and group IV, which consisted of 17, 7, 16 and 13 cultivars, respectively (Table 2).

To distinguish the 53 tobacco varieties by using a set of relatively small number of primers, rather than a cluster analysis of tobacco cultivars with primers, a detailed discrimination process of 53 to-

Table 1. The SCAR and RAPD primers used in the study

Molecular marker	Primer	Sequence	Annealing temperature (°C)	Product size (bp)
SCAR	S1-F	TCTGTGACTGGTGAGTACTC	52	617
	S1-R	GTCTGCTCCCGGCATCCGC		
	S2-R	CACCCGCTGACGCGCCCTGACG	59	752
	S2-F	GGTGTCACGCTCGTCGTTTGGTA		
	S3-R	ATCACCGAAACGCGCGAGACG	58	886
	S3-F	GAAGGGCCGAGCGCAGAAGTGG		
	S4-R	GGTGCACTCTCAGTACAATCTGC	58	525
	S4-F	CCAGTTCGATGTAACCCACTCG		
	S5-R	TTTTCGGGGAAATGTGCGCGG	50	717
	S5-F	TCATTCTGAGAATAGTGTATGC		
	S6-R	TTGTCAGAAGTAAGTTGGCCGC	55	539
	S6-F	CACTTTTCGGGGAAATGTGCGC		
	S7-R	AGTCCCAGACTCAGGAGGGGTGC	59	730
	S7-F	GGTAGGCAAGGACACCACCCAA		
	S8-R	GATTTTGGGTCCCCGGAATCCCG	65	374
	S8-F	GTGGTGACACTCGTCATATAGC		
RAPD	S29	CCCGAGAAAC	36	244–2795
	S32	GGCTCTGGGT	36	244–2484
	S42	TTCCCGCACT	36	335–2500
	S114	TGTGCCTGAC	36	655–2759
	S132	AGGCCGGTAC	36	656–2615
	S301	GAAACGGGTG	36	455–3126
	L189	CACGGCTGCG	36	400–2459

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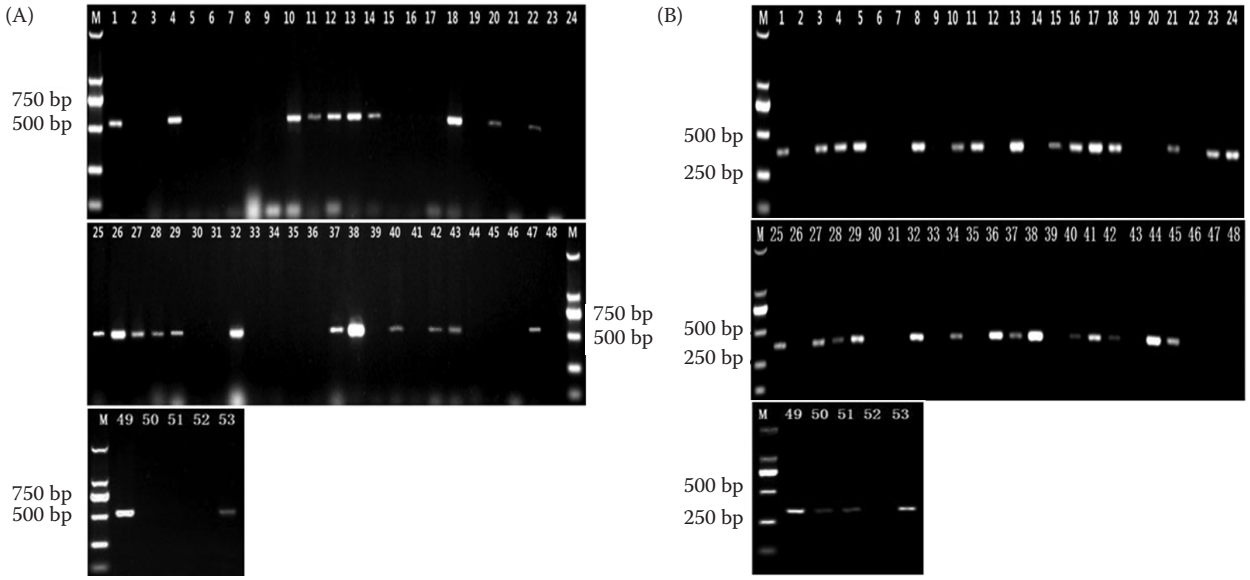

Figure 1. The PCR products of the 53 tobacco cultivars using the SCAR primers S4 (A) and S8 (B)
Lanes 1–53 are the tobacco cultivars listed in Table S1 (in ESM); M indicates the DL2000 DNA ladder

bacco cultivars is presented. A set of 200 RAPD primers were screened, out of which seven primers produced clear and reproducible polymorphic bands that were used to discriminate the tobacco cultivars in each group (Table 1). For group I, 17 tobacco cultivars could be discriminated by the RAPD profiles produced by primers S29, S32 and S132 (Figure S1 and Table S2 in the ESM). Similarly, the tobacco cultivars in Group II by S29 (Figure S2 and Table S3 in ESM), group III by S32, S29 and S114 (Figure S3 and Table S4 in ESM), group IV by S29, S42, S301 and L189 (Figure S4 and Table S5 in ESM) could be discriminated by the selected seven RAPD primers. In conclusion, within the 53 tobacco cultivars, each tobacco cultivar could be first assigned to 1 of 4 groups by 2 SCAR markers, and then identified by the specific RAPD primers for the specific group (Figure 2). Thus, all of the 53 tobacco cultivars were efficiently discriminated by this two-step fingerprinting strategy using only 2 SCAR and 7 RAPD primers.

Through the extensive selection of polymorphic primers, RAPD fingerprinting could be used to identify tobacco cultivars by using producible primers in optimised PCR systems (Chang et al. 2004). Previous studies showed that at least sixteen primers were needed to identify less than 30 tobacco cultivars (Xu et al. 1998; Wang et al. 2003; Yang et al. 2006), which indicated a low level of genetic diversity between the cultivated tobacco cultivars (Rossi et al. 2001; del Piano et al. 2014). The tedious procedure of using more than ten primers could impede the assessment of the genetic diversity of tobacco cultivars. Previous studies showed the potential of using SCAR markers that were developed from RAPD in the identification of the small number of tobacco cultivars (Milla et al. 2005; Ma et al. 2012). In this study, we improved the RAPD fingerprinting by first dividing the large

Table 2. Tobacco cultivar groups divided by the SCAR markers S4 and S8

Group	S4	S8	Tobacco cultivars
I	1	1	Yunyan 85, Yunyan 98, Yunyan 203, Yunyan 317, Yanshai 1, Yanshai 6, Jiyan 1, Jiyan 7, Jiyan 9, Jiyan 10, Yuyan 10, Longjiang 911, Longjiang 925, Longjiang 982, K346, SAM, 9407
II	1	0	Honghuadajinyuan, Yanshai 2, Yanshai 8, Yanshai 10, Jiyan 5, NC71, Cuibi 1
III	0	1	Yunyan 97, Yunyan 99, Yunyan 116, Yanshai 3, Yanshai 4, Yanshai 5, Yanshai 9, Yiyan 1, Yanan 1, Yuyan 12, Zhongyan 206, K326, NC89, NC102, Qinyan 96, V2
IV	0	0	Yunyan 87, Yunyan 100, Yunyan 105, Yunyan 202, Yanshai 7, Yuyan 6, Yuyan 7, Yuyan 11, Zhongyan 100, Longjiang 981, NC297, CT1410, GR17

1 – present; 0 – absent

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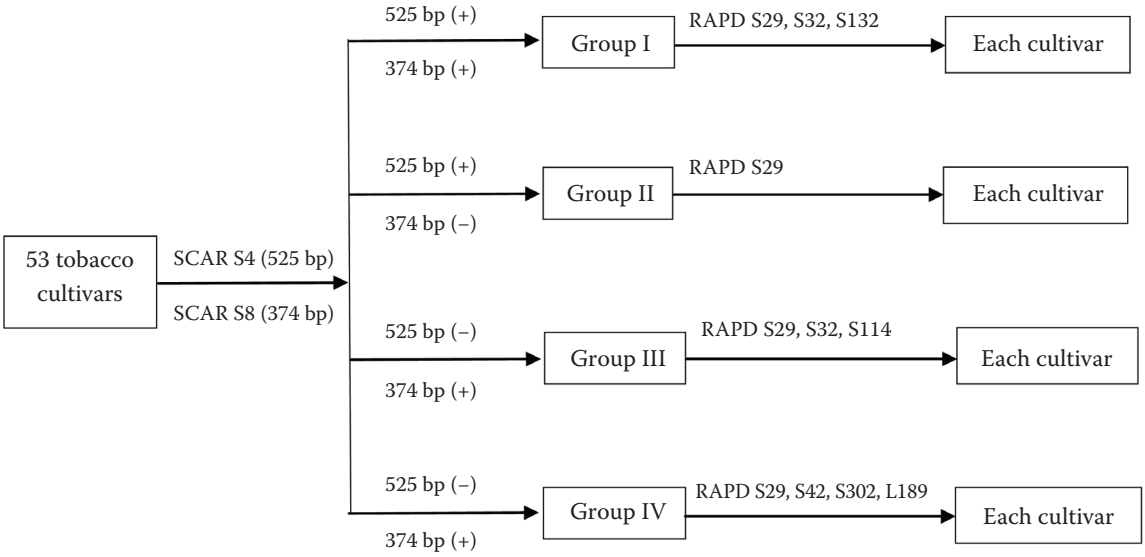

Figure 2. Flow diagram showing the discrimination of the 53 tobacco cultivars using the SCAR and RAPD markers

number of cultivars into small groups by 2 SCAR markers, and then we successfully discriminated 53 tobacco cultivars by using only 7 RAPD primers. It was suggested that the two-step SCAR-RAPD fingerprinting strategy could be used to develop an inexpensive and efficient method to identify massive cultivars in tobacco or other crops.

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