Development of Sequence-characterized Amplified Region Markers for the Identification of Grapevine Cultivars

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Abstract. Grapevine cultivars have traditionally been identified based on the morphological characteristics, but the identification of closely related cultivars has been difficult because of their similar pedigree backgrounds. In this study, we developed DNA markers for genetic fingerprinting in 37 grapevine cultivars, including 20 cultivars bred in Korea. A total of 180 randomly amplified polymorphic DNA (RAPD) markers were obtained using 30 different primers. The number of polymorphic bands ranged from three (OPG-08 and OPU-19) to nine (OPV-01 and UBC116), with an average of six. RAPD markers were used in cluster analysis performed with the unweighted pair-group method of arithmetic averages (UPGMA). The average similarity value was 0.69 and the dendrogram clustered the 37 grapevine cultivars into five clusters. The relationships among the grapevine cultivars were consistent with the known pedigrees of the cultivars. The 50 RAPD fragments selected were sequenced for the development of sequence-characterized amplified region (SCAR) markers. As a result, 16 of 50 fragments were successfully converted into SCAR markers. A single polymorphic band, the same size as the RAPD fragments or smaller, was amplified depending on the primer combinations in the 14 SCAR markers, and codominant polymorphisms were detected using the SCAR markers G119_412 and GB17_732. Among these markers, combination of 11 SCAR markers, GG05_281, G116_319, G146_365, G119_412, GW04_463, G169_515, G116_539, GV04_618, GV01_678, GG05_689, and GB17_732, provided sufficient polymorphisms to distinguish the grapevine cultivars investigated in this study. These newly developed markers could be a fast and reliable tool for identifying grapevine cultivars.

Grapevine (*Vitis* spp.) belongs to the Vitaceae family and is one of the most economically important perennial crops in the world. The number of global cultivars has been estimated to be between 5000 and 8000 and many have been cultured for several centuries (Schneider et al., 2001). The broad geographic expansion of the cultures has caused a problem in identifying cultivars, especially given their synonyms and homonyms (Zhao et al., 2011). Grapevine cultivars have traditionally been identified based on ampelography, which is the analysis and comparison of morphological characteristics of leaf shape, fruit clusters, and berries (Galet, 1979). However, phenotypic observations are affected by environmental conditions and cultural management, making it impossible to distinguish between very close genotypes (Meneghetti et al., 2011). There-

fore, precise, fast, and reliable tools are required for the protection of plant varieties and for the practical breeding purpose. Polymerase chain reaction (PCR)-based DNA markers have become useful for identifying cultivars, analyzing parentage studies, and evaluating genetic diversity.

Many comparative investigations have been carried out to explore which technique is the most suitable and reliable for identifying cultivars (Powell et al., 1996). The choice of a DNA marker depends on the scale and purpose of identifying the cultivar. Several different types of DNA markers have been applied to identify grapevine cultivars and analyze their genetic relationships (Castro et al., 2012; Guo et al., 2012; This et al., 2004). Simple sequence repeats (SSR) markers provide excellent DNA fingerprinting methods because of their codominant Mendelian inheritance, high degree of polymorphisms, and abundance in genomes (Sefc et al., 1999). However, the major drawbacks of SSR markers are the time and cost involved for developing species-specific primer pairs. In addition, SSR assays require high-resolution gels (Gianfranceschi et al., 1998). Among DNA-based markers, RAPD markers are easy, simple, and inexpensive, and they do not require any previous se-

quence information. RAPD markers are used as single primers of arbitrary nucleotide sequences (10–12 mer) to amplify anonymous PCR fragments from genomic DNA. The use of short primers and low annealing temperatures makes RAPD markers extremely sensitive to reaction conditions, resulting in low reproducibility among different laboratories (Goulão et al., 2001). RAPD has been restricted in practical applications because of poor reproducibility and its competitive priming. To overcome these problems, longer primers have been developed from RAPD fragment sequences by Paran and Michelmore (1993). Longer primers generate a SCAR, making it possible to amplify only the target DNA fragment by PCR. After converting RAPD markers into SCAR markers, the specificity and stability can be greatly improved. SCAR markers have been developed in many fruit species including apple and grapevine (Vidal et al., 2000; Xu et al., 2001). We have also reported previously that SCAR markers are useful for effectively distinguishing apple cultivars (Cho et al., 2010). In Korea, 20 grapevine cultivars have been released by the National Institute of Horticultural and Herbal Science (NIHHS) of the Rural Development Administration, the Horticulture Research Section, and the Grape Research Institute from 1993 to 2012. In this study, a reliable PCR-based technique was investigated for the identification of grapevine cultivars, including those bred in Korea. We clearly demonstrated that 16 SCAR markers developed from RAPD marker analyses could be used for effectively identifying 37 different grapevine cultivars.

Materials and Methods

Plant materials and DNA isolation. A total of 37 grapevine cultivars, including the newly released cultivars Saemaru and Sweet Dream as well as introduced cultivars, were used for genomic DNA analysis (Table 1). All plants were maintained at the NIHHS, Wanju, Korea. Genomic DNA was extracted from the young leaves of the grapevines using the DNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA). We determined the concentration and purity of the DNA preparation by using a NanoDrop (Thermo Scientific, Rockford, IL) and 0.8% agarose gel electrophoresis, respectively. A working solution of 5 ng·μL⁻¹ of the genomic DNA was prepared for PCR analysis.

RAPD analysis. To identify suitable primers for this study, four cultivars (Alden, Campbell Early, Heukboseok, and Kyoho) were tested with 30 Operon (Operon Technologies, Alameda, CA) and University of British Columbia (UBC, Vancouver, BC, Canada) primers. The 30 PCR primer pairs were selected based on their ability to produce clearly and repeatedly polymorphic bands among four cultivars. These selected primers were used to obtain genotype-specific RAPD markers. PCR reactions for RAPD were performed in a 12.5-μL reaction mixture containing 20 ng template DNA,

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1× PCR buffer, 0.36 μM random primer, 200 μM of each deoxyribonucleotide triphosphate (dNTPs; dATP, dTTP, dGTP, and dCTP), 3 mM MgCl₂, and 0.5 units of *Taq* DNA polymerase (Genetbio, Daejeon, Korea). Amplifications were carried out in a thermal cycler (C1000, Bio-Rad Laboratories, Hercules, CA) with cycle parameters as follows: an initial denaturation at 94 °C for 4 min, 10 cycles of denaturation at 94 °C for 45 s, 37 °C for 45 s, and 72 °C for 2 min, and then 35 cycles of denaturation at 94 °C for 45 s, 42 °C for 45 s, and 72 °C for 2 min. The final extension was at 72 °C for 10 min. Amplification products were analyzed on 1.4% agarose gel electrophoresis in 0.5× Tris-borate-EDTA (TBE; 45 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer at 150 V for 3 h, and visualized by ultraviolet illumination after ethidium bromide staining. Since the reproducibility of the described polymorphisms is essential for a guaranteed certificate of accuracy, we selected bands that were consistently produced in two different RAPD analyses. For the genetic relationship analysis, the amplified bands for all the individuals were scored as either 1 (present) or 0 (absent). Genetic similarity was estimated using a simple-matching coefficient (Sneath and Sokal, 1973) and multivariate statistical package program version 3.13 (Kovach Computing Services, Wales, UK). A dendrogram was constructed by cluster analysis using UPGMA.

Cloning and sequencing of specific RAPD fragments markers. Selected cultivar-specific RAPD bands were excised from 1.4% agarose gel with a sterile cutter. The extracts were purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR2.1-TOPO vector by a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). After cloning, five white colonies from each transformation were selected and cultured overnight in 5 mL Luria-Bertani liquid medium (Tryptone 10 g·L⁻¹, NaCl 5 g·L⁻¹, and yeast extract 5g·L⁻¹) containing 100 μg·mL⁻¹ ampicillin. Plasmid DNAs were isolated using a QIAprep-spin Plasmid Miniprep Kit (Qiagen, Hilden, Germany) and sequenced on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

Based on the sequence data, specific primers (18–27 mer) were designed using Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3-plus/primer3plus.cgi/>). Some of these primers included the original 10-mer sequence of the RAPD primer used for amplification. For each RAPD marker, four oligonucleotides were designed to be used as SCAR primers. PCR was used to amplify cultivar-specific bands in a 15-μL reaction mixture of 20 ng template DNA, 1× PCR buffer, 0.5 μM forward and reverse primer, 200 μM of each dNTP, and 0.5 units of Hot Start *Taq* DNA polymerase (Genetbio, Daejeon, Korea). The amplifications were performed with an initial denaturation at 94 °C for 10 min, followed by 30 cycles at 94 °C for 30 s, 58–63 °C for 30 s, and 72 °C for 1 min, and the final extension was at 72 °C for 5 min. The SCAR products were resolved electrophoretically in a 1.4% agarose gel.

Table 1. Grapevine cultivars used in this study including their parentage and origins.

No.	Cultivar	Parentage	Origin
1	Alden	Ontario × Grosse Guillaume	United States
2	Beniizu	Beni Fuji mutation	Japan
3	Black Eye	<i>Vitis amurens</i> × Muscat Bailey A	Korea
4	Black Olympia	Kyoho × Kyogei	Japan
5	Black Star	Honey Black × Campbell Early	Korea
6	Campbell Early	Moore Early × (Belvidere × Muscat Hamburg)	United States
7	Cheonghyang	Red Pearl × Muscat Bailey A	Korea
8	Cheongsoo	Seibel 9110 × Himrod Seedless	Korea
9	Delaware	(<i>Vitis labrusca</i> × <i>Vitis aestivalis</i>) × <i>Vitis vinifera</i>	United States
10	Doonuri	Schuyler × Campbell Early	Korea
11	Dutchess	Concord × Montgomery	United States
12	Golden Muscat	Muscat Hamburg × Diamond	United States
13	Green King	Tensyu × Benifuji	Korea
14	Heukboseok	Beniizu × Kyoho	Korea
15	Heugsoosul	Golden Muscat × Pione	Korea
16	Himrod Seedless	Sultanina × Ontario	United States
17	Hongaram	Tano Red × Muscat Hamburg	Korea
18	Hongdan	Campbell Early × Himrod Seedless	Korea
19	Hongisul	Campbell Early × Himrod Seedless	Korea
20	Hongsodam	Dutchess × Daepyeong Dela	Korea
21	Jarang	Chance seedling	Korea
22	Kyoho	Centennial × Ishihara Wase	Japan
23	Muscat Bailey A	Bailey × Muscat Hamburg	Japan
24	Muscat Hamburg	Schiava Grossa × Muscat of Alexandria	UK
25	Narsha	Alden × <i>Vitis amurens</i>	Korea
26	Okrang	Sheridan × Campbell Early	Korea
27	Pione	Kyoho × Muscat Cannon Hall	Japan
28	Saemaru	Sheridan open pollination	Korea
29	Schuyler	Zinfandel × Ontario	United States
30	Seibel 9110	Seibel 5455 × Seibel 4938	France
31	Suok	Kyoho × Beniizu	Korea
32	Super Hamburg	Campbell Early × Muscat Hamburg	Japan
33	Sweet Dream	Honey Black × Campbell Early	Korea
34	Tamnara	Campbell Early × Himrod Seedless	Korea
35	Tankeumchu	Tano Red × Super Hamburg	Korea
36	Tano Red	Unknown	Japan
37	<i>Vitis amurens</i> Rupr.	—	—

Table 2. Randomly amplified polymorphic DNA primers used in this study, their sequences, and their numbers of polymorphic fragments produced.

No.	Primer	Sequence	No. of polymorphic fragments produced
1	OPA-07	GAAACGGGTG	5
2	OPB-17	AGGGAACGAG	6
3	OPE-09	CTTCACCCGA	6
4	OPE-17	CTACTGCCGT	5
5	OPG-05	CTGAGACGGA	5
6	OPG-06	GTGCCTAACCC	5
7	OPG-08	TCACGTCCAC	3
8	OPG-17	ACGACCGACA	7
9	OPK-08	GAACACTGGG	8
10	OPK-10	GTGCAACGTG	6
11	OPK-11	AATGCCCCAG	7
12	OPK-20	GTGTGCGGAG	8
13	OPL-02	TGGGCGTCAA	5
14	OPT-12	GGGTGTGTAG	5
15	OPT-16	GGTGAACGCT	6
16	OPU-03	CTATGCCGAC	4
17	OPU-13	GGCTGGTTCC	5
18	OPU-17	ACCTGGGGAG	6
19	OPU-19	GTCAGTGCGG	3
20	OPV-01	TGACGCATGG	9
21	OPV-04	CCCCTCACGA	5
22	OPW-04	CAGAAGCGGA	6
23	UBC116	TACGATGACG	9
24	UBC119	ATTGGGCGAT	8
25	UBC146	ATGTGTTGCG	6
26	UBC213	CAGCGAATA	6
27	UBC169	ACGACGTAGG	7
28	UBC427	GTAATCGACG	8
29	UBC507	AGACGTACTC	6
30	UBC509	ACAGAGACTG	5

Results and Discussion

Screening of specific RAPD fragments. Thirty random primers used in the RAPD analysis showed different polymorphic bands in 37 grapevine cultivars. In total, 180 distinct markers were obtained from the RAPD analysis. The results obtained for each random

primer are summarized in Table 2. Unambiguous amplified DNA bands were carefully chosen and scored for cultivar identification to ensure the absence of artifacts. The number of polymorphic bands varied from three to nine, and six bands per primer were generated on average. The size of the scorable amplified fragments ranged from 200 to

2000 base pairs (bp). Six random primers (OPK-08, OPK-20, OPV-01, UBC116, UBC119, and UBC427) generated eight to nine discrete reproducible bands, whereas OPG-08 and OPU-19 primers resulted in three polymorphic bands. An example of the RAPD patterns generated with the UBC119 primer is shown in Figure 1. An ≈ 440 bp

Fig. 1. Randomly amplified polymorphic DNA profiles of 37 grapevine cultivars amplified with UBC119 primer. Lane numbers represent grapevine cultivars as shown in Table 1. M, 100 base pairs plus DNA ladder.

Fig. 2. Dendrogram of 37 grapevine cultivars based on genetic similarity values obtained from the randomly amplified polymorphic DNA data. Scale shown below indicates genetic similarity values. Numbers above branches correspond to genetic similarity values of nodes and square brackets mean clusters.

polymorphic band was amplified from eight Korean grapevine cultivars (Cheongsoo, Green King, Heukboseok, Heukgoosul, Hongarm, Jarang, Suok, and Sweet Dream) and 10 introduced grapevine cultivars (e.g., Beniizu, Golden Muscat, and Super Hamburg). Vidal et al. (1999) reported that 33 primers produced 144 polymorphic RAPD markers with an average of 4.4 bands per primer among the 32 white grapevine varieties. El-Sayed et al. (2011) obtained 66 reproducible fragments using six selected primers among the eight grapevine cultivars. The different number of polymorphic bands selected might account for the genetic diversity in the grapevine cultivars. RAPD analysis can be used to identify many useful polymorphisms rapidly and efficiently, and has tremendous potential for cultivar identification (Lu et al. 1996). However, this technique is sensitive to experimental conditions, as RAPD analysis commonly uses nonstringent PCR conditions (Muralidharan and Wakeland, 1993). The lack of reproducibility is a pitfall both to cultivar identification in routine procedures and to data exchange among laboratories (Büscher et al., 1993). Despite a number of drawbacks, RAPD markers are still widely used for identifying cultivars and clones and for genetic analysis (El-Sayed et al., 2011; Karatas and Ağaoğlu, 2010; Zhao et al., 2011). Zhao et al. (2011) created a cultivar identification diagram of 69 grapevine cultivars obtained with seven RAPD markers after they confirmed

the reproducibility of the analytic conditions. Among the polymorphic bands, 50 amplified DNA bands were selected as RAPD markers for identifying the cultivars and were used in subsequent analyses.

Cluster analysis based on RAPD markers. Genetic relationships among the 37 grapevine cultivars based on their genetic distances were clustered using UPGMA in a dendrogram shown in Figure 2. The genetic similarity coefficient values ranged from 0.52 to 0.96. The average similarity value was 0.69 and the 37 grapevine cultivars were divided into five clusters. *Vitis amurensis* Rupr., a species of grapevine native Asian continent, was in Cluster I. Cluster II consisted of seven cultivars including three Korean cultivars. Cheongsoo and Honsodam cultivars are the offspring of Seibel 9110 and Dutchess, respectively, and they clustered together (Fig. 2). Yoo et al. (2009) reported that Cheongsoo and its seed parent Seibel 9110 cultivars were grouped in the same cluster by RAPD analysis. Cluster III comprised Muscat Hamburg, Muscat Bailey A, and two Korean newly bred cultivars, Hongarm and Black Eye which are the offspring of Muscat Hamburg and Muscat Bailey A cultivars, respectively. Cluster IV comprised 22 cultivars and most of them are related to Campbell Early, Kyoho, and Himrod Seedless cultivars. The cultivars Hongdan, Tamnara, and Hongisul which have the same pedigree of Campbell Early and Himrod Seedless cultivars were clustered together.

The Black Olympia cultivar is an offspring of Kyoho cultivar and was clustered with it as previously reported (Guo et al., 2012). Cluster V consisted of Alden, Narsha, and Okrang cultivars. The Narsha cultivar is an offspring of Alden and *V. amurensis* Rupr. and was clustered with Alden cultivar together. Most of the grapevine cultivars except Pione cultivar having common parents were clustered together in the dendrogram. These results reflected the close relationship between Korean bred cultivars and Euro-America hybrid table grapevine cultivars.

Development of SCAR markers derived from RAPD markers. The sequences of the 50 polymorphic fragments (<1000 bp) identified in this study were determined to convert the RAPD markers into SCAR markers. Based on these sequence data, we synthesized specific primer sets to amplify the internal polymorphic fragment region. Of the 50 reproducible RAPD markers, 16 were successfully converted to SCAR markers. Some primer sets resulted in a failure of PCR amplification, whereas others produced unclear polymorphisms (data not shown). The developed SCAR primer sequences, annealing temperature, and amplicon size are summarized in Table 3. The sequence results showed that the UBC507_334 RAPD fragment had a size of 334 bp and contained the original sequences of the UBC507 primer at the ends of the cloned RAPD fragment. These results clearly demonstrated that the cloned fragment

Table 3. Sixteen sequence-characterized amplified region (SCAR) primer pairs derived from cloned randomly amplified polymorphic DNA (RAPD) markers sequences.

RAPD marker	SCAR marker	Primer sequence (5'→3')	Annealing temp (°C)	Amplicon size (bp ^a)
UBC509_243	G509_243	F ^b : ACAGAGACTGCCAACATATGAGTT R: ACAGAGACTGGTACTACAAGTGAA	60	243
OPG05_281	GG05_281	F: CTGAGACGGACGAAATGAGATTAT R: CTGAGACGGAGTTATCCCGAATCA	63	281
UBC116_331	G116_319	F: CGGCCATACATGTACAGAAATAAG R: ATGACGCACAGTGAGGTAGATATT	63	319
UBC507_334	G507_334	F: AGACGTACTCTCTCATGCATCTTT R: AGACGTACTCAACAAATTTAACAT	63	334
UBC427_348	G427_348	F: GTAATCGACGTCACACATAAC R: GTAATCGACGGTGGACTAC	63	348
UBC146_365	G146_365	F: ACGACGTAGGGGCATATCTGCTGG R: ACGACGTAGGCGGACAAAATTTCT	63	365
OPU13_380	GU13_380	F: GGCTGGTTCCACCGGCTCCTTCAG R: GGCTGGTTCTGACTAAAATTTGT	63	380
UBC119_443	G119_412	F: ATGATTGAAATAAGCCCAAAAGAG R: ATTGGGCGATCGTCTGTACAATGA	63	337, 412
OPV01_428	GV01_424	F: TGACGCATGGCACAGAACAAATTTA R: GCATGGCGAGTAGATACAACATA	63	424
OPW04_463	GW04_463	F: CAGAAGCGGACAGCTTGTACCAGG R: CAGAAGCGGAATATGAGGCCATCC	63	463
UBC169_515	G169_515	F: ACGACGTAGGCAAGATGTCAAACC R: ACGACGTAGGTAGTAAACAACCTCT	63	515
UBC116_539	G116_539	F: TACGATGACGATCATGTCCGGAAG R: TACGATGACGACAACCCGAGATGT	60	539
OPV04_618	GV04_618	F: ACGACGTAGGTAGTAAACAACCTCT R: CCCCTCACGATCGAGAATATTTA	63	618
OPV01_678	GV01_678	F: TGACGCATGGGTAACCAAGTTCAAC R: TGACGCATGGCAAATAAATCAACC	58	678
OPG05_689	GG05_689	F: CTGAGACGGAGATGGGGAGCTCAA R: CTGAGACGGATCAATCACCCAACC	63	689
OPB17_732	GB17_732	F: AGGGAACGAGGACAACAAGACATTGAT R: AGGGAACGAGCAAGAGCGTTGACGTGG	63	732, 793

^abp = base pair.

^bF = forward; R = reverse.

was derived from the amplified RAPD product. The SCAR marker derived from the UBC507_334 RAPD fragment was designated G507_334. Compared with the RAPD marker, the G507_334 marker amplified using a specific primer set was more useful because only the differential DNA was amplified, which made it easy to identify the grapevine cultivars.

A 334-bp fragment was amplified in the Cheonghyang, Delaware, Dutchess, Hongsodam, and Muscat Bailey A cultivars using the G507_334 SCAR marker (Table 4; Fig. 3A). A single polymorphic band of the same size as the RAPD fragment or smaller was amplified by a combination of primers in the 14 SCAR markers. As expected from the sequence data, the GU13_380 SCAR marker produced a single 380-bp fragment in the Hongaram, Jarang, Muscat Hamburg, Okrang, and Super Hamburg cultivars. The GV01_424 and G116_539 SCAR markers were produced only in the Narsha and Pione cultivars, respectively (Fig. 3B). The SCAR markers G119_412 and GB17_732 showed codominant polymorphisms in Cheonghyang and Muscat Bailey A, and Kyoho cultivars, respectively, depending on the primer combinations, as shown previously (Paran and Micheltore, 1993; Fig. 3C and D). Amplification of the targeted bands on additional cultivars is the main problem when using SCAR primers (Vidal et al., 2000). However, the size of each amplified band differs from each other and could be also applicable as an identification marker (Kim et al., 2000). The G119_412 SCAR marker produced a 412-bp band in cultivars Beniizu, Black Eye, Black Olympia, Cheonghyang, Cheongsoo, Delaware, Golden Muscat, Green King, Heukboseok, Heukgoosul, Himrod Seedless, Hongaram, Jarang, Kyoho, Muscat Bailey A, Muscat Hamburg, Pione, Seibel 9110, Suok, Super Hamburg, Sweet Dream, Tano Red, and *V. amurensis* Rupr. In addition, another band appeared at the 337-bp band in Muscat Bailey A and its offspring Cheonghyang cultivars. In addition, the GB17_732 SCAR marker produced two bands of 732 and 793 bp. A 732-bp fragment was amplified in 19 cultivars including Doonuri, and a 793-bp fragment was amplified in five cultivars including Beniizu. Two bands were generated only in Kyoho cultivar. The Heukboseok and Suok cultivars generated a single 732- and 793-bp fragment, respectively. Their parents were the same as Beniizu and Kyoho. Therefore, the GB17_732 SCAR marker will be more useful for distinguishing between these cultivars. The developed 16 SCAR markers generated four (Dutchess and Heukboseok cultivars) to 10 (Hongarm and Okrang cultivars) bands in the 37 grapevine cultivars (Table 4). Alden cultivar had five bands of G509_243, GG05_281, G427_348, GW04_463, and GB17_732-1 SCAR markers. A subset of smaller markers is needed for increased efficiency in cultivar identification. Applying 11 SCAR markers (GG05_281, G116_319, G146_365, G119_412, GW04_463, G169_515, G116_539,

GV04_618, GV01_678, GG05_689, and GB17_732) was sufficient to distinguish the 37 grapevine cultivars according to the number and size of PCR product bands.

DNA markers must be reliable, practical, and validated in different laboratories for use in fingerprinting. Inconsistencies in the results of RAPD reactions make RAPD markers less useful than desired (Xu et al., 1995). Bernet et al. (2003) reported that only reproducible RAPD bands should be selected under high-resolution electrophoresis and that they should be cloned and sequenced to allow for the design of specific primers. Furthermore, SCAR markers often fail to reveal original polymorphisms because the original RAPD polymorphisms are caused by mismatches in one or more nucleotides at the priming sites. These mismatches could be overcome by longer primers (Paran and Micheltore, 1993; Xu et al., 1995). SCAR markers with clear

polymorphisms are derived from RAPD polymorphisms caused by differences in the nucleotide sequences at the priming sites or by structural rearrangements within the amplified sequence (Paran and Micheltore, 1993). Various methods have been used to investigate additional polymorphisms, such as increasing annealing temperature, the use of longer SCAR primers, and restriction enzyme treatment, but polymorphisms could not be easily recovered (Paran and Micheltore, 1993; Xu et al., 1995).

Of the 50 RAPD markers sequenced, 16 SCAR markers were developed in this study. The low percentage of SCAR marker conversion (32%) was presumably caused by the selection of polymorphic RAPD bands that contained multiple fragments of identical molecular weight in the ethidium bromide-stained agarose gels (Paran and Micheltore, 1993). Similar results have been reported for apple (Cho et al., 2010) and grapevine cultivars (Vidal et al., 2000). Five putative

Table 4. Result of polymerase chain reaction using 16 sequence-characterized amplified region (SCAR) markers and 37 grapevine cultivars.

SCAR marker	Cultivar no. ²																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
G509_243	1 ^y	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
GG05_281	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0
G116_319	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0
G507_334	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0
G427_348	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1
G146_365	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
GU13_380	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
G119_412-1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
G119_412-2	0	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0	0
GV01_424	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GW04_463	1	1	0	1	1	1	0	1	0	0	0	1	1	0	1	1	1	1	1
G169_515	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1
G116_539	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GV04_618	0	1	1	1	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1
GV01_678	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1	1
GG05_689	0	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1
GB17_732-1	1	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0	0	0	1
GB17_732-2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
No. of SCAR markers	5	8	6	6	6	9	8	8	7	6	4	7	5	4	9	9	10	6	8
SCAR marker	Cultivar no.																		
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
G509_243	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
GG05_281	1	1	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0
G116_319	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1
G507_334	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G427_348	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0
G146_365	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0
GU13_380	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
G119_412-1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G119_412-2	0	1	1	1	1	0	0	1	0	0	1	1	1	1	0	0	1	1	1
GV01_424	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
GW04_463	0	1	1	0	0	1	0	1	0	0	1	1	0	1	1	1	1	1	0
G169_515	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0
G116_539	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
GV04_618	0	1	1	0	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1
GV01_678	1	1	1	0	0	0	1	0	1	0	0	1	1	1	1	1	1	1	1
GG05_689	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
GB17_732-1	1	0	1	1	1	1	1	0	1	1	0	0	1	1	0	1	0	0	0
GB17_732-2	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
No. of SCAR markers	8	7	8	6	7	7	10	6	6	5	5	7	9	8	6	7	9	6	

²Number of cultivars are listed in Table 1.

^yThe amplified band was scored as 1 (present) or 0 (absent).

Fig. 3. Amplified fragment patterns of G507_334 (A), GV01_424 (B), G119_412 (C), and GB17_732 (D) sequence-characterized amplified region (SCAR) markers in 37 grapevine cultivars. Arrows indicate SCAR markers. Lane numbers represent grapevine cultivars as shown in Table 1. M, 100 base pairs plus DNA ladder.

genotype-specific RAPD markers in the grapevine cultivars have been sequenced and only two primer pairs still produced a specific SCAR marker among the 30

sequence-specific primers (Vidal et al., 2000). The main problem when developing SCAR markers by the direct sequencing of RAPD products is reamplified untargeted

sites due to the poor specificity of the short 10-mer primers (Hernández et al., 1999).

In summary, 16 novel SCAR markers were developed by cloning and sequencing of cultivar-specific RAPD bands. We clearly showed that a subset of smaller markers was sufficient to identify cultivars with a high level of accuracy. These SCAR markers have advantages for large-scale analyses because of their low cost, high reproducibility, and ease of use without elaborate electrophoresis. The SCAR marker system developed in this study offers great potential for protecting intellectual property rights and allowing the certification of vegetatively propagated grapevine cultivars.

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