Efficiency of Randomly Amplified Polymorphic DNA to Sequence Characterized Amplified Region Marker Conversion and their Comparative Polymerase Chain Reaction Sensitivity in Cucumber

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ABSTRACT. The conversion of randomly amplified polymorphic DNA (RAPD) markers to sequence characterized amplified region (SCAR) markers, and the effects of differing polymerase chain reaction (PCR) conditions were studied in cucumber (*Cucumis sativus* L.). Attempts were made to clone and sequence 75 RAPD PCR products to produce SCAR primers (16 to 22 nucleotides) designed to amplify original RAPD PCR products. The influence of template DNA source, purity, and concentration, MgCl₂ concentration, Taq polymerase source, and type of thermocycler upon RAPD and SCAR marker performance was evaluated. Conversion of RAPD to SCAR markers was not universally successful, and SCAR primers reacted differently to varying PCR conditions. Only 48 (64%) of 75 RAPD markers were successfully converted to SCAR markers and 11 (15%) of these reproduced the polymorphism observed with the original RAPD PCR product. Moreover, some SCAR primer pairs produced multiple polymorphic PCR products. The band intensity of SCAR markers were brighter ($P = 0.05$) than their corresponding RAPD markers with only one exception. The SCAR markers examined were less influenced ($P = 0.05$) by MgCl₂ concentration than their corresponding RAPD markers. However, some SCAR markers were more sensitive to reaction impurities than their RAPD counterparts and SCAR markers tended to be less readily visualized (decrease in frequency of visible PCR product) with low concentrations (1 and 2 mM) of template DNA than their corresponding RAPD markers. Neither the source of Taq nor the type of thermocycler used affected the performance of SCAR and RAPD markers. These data suggest that although SCAR markers may demonstrate enhanced performance over the RAPD markers from which they are derived, careful consideration must be given to both the costs and potential benefits of SCAR marker development in cucumber.

Genetic markers have assisted in genomic characterization of various crop species (Rafalski and Tingey, 1993). Although other genetic marker systems are used, most rely upon DNA sequence polymorphisms (Staub et al., 1996a). Each system has unique advantages and disadvantages based on cost, experimental objectives, and type of information desired. Regardless of its inherent methodology, the effective deployment of any marker system for genetic analysis requires that it be reliable and consistent.

The random amplified polymorphic DNA (RAPD) (Williams et al., 1990) or arbitrarily primed polymerase chain reaction (APPCR) (Welsh and McClelland, 1990) marker system identifies DNA sequence polymorphisms, is relatively inexpensive, and is amenable to high through-put investigation. With this molecular marker system, segments of genomic DNA are amplified in a polymerase chain reaction (PCR) (Mullis et al., 1986) with random oligodeoxynucleotides as primers (Williams et al., 1990) followed by agarose gel electrophoresis. RAPD markers are usually dominant and polymorphisms are observed as the presence or the absence of DNA bands of a particular size.

A problem with RAPD analysis, however, is its low tolerance

to methodological variations. Thus, PCR reaction conditions must be as close to identical as possible to obtain consistent results. Even minute changes in PCR reaction components can lead to variation that will produce erroneous results (Hedrick, 1992; Lopez-Buesa, 1994; Staub et al., 1996a; Weeden et al., 1992).

The inherent sensitivity of the RAPD methodology has led to the development of other PCR-based molecular marker systems that are more tolerant of minor variation in PCR reaction conditions. One such proposed PCR-based marker system is sequence characterized amplified region (SCAR) methodology (Paran and Michelmore, 1993; Rafalski and Tingey, 1993). A SCAR marker is developed from a RAPD marker by sequencing both ends of the PCR product and then designing longer (usually ≈20 nucleotides) PCR primers.

One significant advantage of SCAR markers versus RAPD markers, is the production of unique (single band) PCR products that are less influenced by reaction conditions. Some well-documented conditions known to influence PCR performance include MgCl₂ concentration, template DNA (purity, concentration and source), Taq polymerase source, and thermocycler (Boleda et al., 1996; Kocher and Wilson, 1991; Staub et al., 1996b; Weeden et al., 1992; Wolff et al., 1993). Since they anneal to longer specific sequences of the template DNA, they should be more tolerant to PCR condition variation. However, direct comparisons between SCAR and RAPD marker performance have not been reported.

We have used RAPD markers in cucumber for linkage analysis (Serquen et al., 1997) and diversity assessment (Horejsi, 1998). However, RAPD markers are susceptible to variation in type and concentration of reagents during the PCR (Staub et al., 1996b). Thus, the conversion of RAPD to SCAR markers has been recommended (Paran and Michelmore, 1993) to increase the reliability

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of PCR markers in this species. To test this recommendation a study was designed for cucumber to determine 1) the efficiency of RAPD to SCAR conversion and 2) to compare the performance of SCAR and RAPD markers under differing PCR conditions. Experiments examined the effect of template DNA type, purity, and concentration, $MgCl_2$ concentration, Taq polymerase source, and thermocycler type. Information on the conversion efficiency and reaction performance of SCAR markers will provide a basis for determining the potential utility of this marker methodology for breeding and germplasm management in cucumber.

Materials and Methods

DNA EXTRACTION. DNA was extracted from leaf tissue using a standard CTAB phenol/chloroform procedure developed for cucumber (Sambrook et al., 1989; Serquen et al., 1997; Staub et al., 1996b). Leaf tissue samples consisted of bulks from twelve 2-week-old seedlings. The DNA was quantified using a Hoefer TKO 100 minifluorometer (Hoefer Scientific Instruments, San Francisco, Calif.) following the manufacturer's protocol and stored at $-20^{\circ}C$.

RAPD MARKERS. The standard RAPD PCR protocol employed was similar to that of Williams et al. (1990) as modified by Staub et al. (1996b). The 15 mL volume PCR contained 3.0 mM $MgCl_2$, 0.2 mM dNTPs (0.05 mM of each: dATP, dGTP, dTTP and dCTP), 15 ng DNA, 0.3 mM primer, commercial polymerase buffer and 1 unit Taq DNA polymerase. The $MgCl_2$, polymerase buffer, dNTPs, and Taq polymerase were purchased from Promega (Madison, Wis.). The RAPD primers were purchased from Operon Technologies (Alameda, Calif.) and the University of British Columbia, Vancouver, Canada. PCRs were performed in polyethylene tubes in a Perkin Elmer (Foster City, Calif.) GeneAmp PCR System 9600 thermocycler using the following cycling profile: $94^{\circ}C/4$ min; 3 cycles of $94^{\circ}C/15$ s, $35^{\circ}C/15$ s, $59^{\circ}C$ ramp to $72^{\circ}C/75$ s; 40 cycles of $94^{\circ}C/15$ s, $40^{\circ}C/15$ s, $59^{\circ}C$ ramp to $72^{\circ}C/75$ s; $72^{\circ}C/7$ min with subsequent cooling to $4^{\circ}C$.

After completion of the PCR, 3 μL of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll) were added to each reaction tube. The samples were loaded onto a 1.6% agarose gel containing $0.5 \mu g \cdot mL^{-1}$ ethidium bromide. Electrophoresis was performed in the gels in TAE buffer [4.84% tris, 1.14% acetic acid, 0.375% EDTA] for 3 h at ≈ 100 v using a BRL H4 apparatus (Life Technologies, Gaithersburg, Md.). The gels were immediately photographed under UV light using an Eagle Eye still video system (Stratagene, LaJolla, Calif.).

SCAR MARKERS. From 71 polymorphic loci detected between cucumber lines G421 and H19 (Serquen et al., 1997) and 4 polymorphic markers linked to *dm* [the downy mildew resistance gene (Horejsi, 1998)], 75 RAPD markers were selected for conversion into SCAR markers.

The procedure for conversion of RAPD to SCAR markers was similar to that described by Paran and Michelmore (1993). RAPD reactions were performed as described above, and the amplified DNA of each target RAPD band was cut from the gel and purified using the Wizard minicolumn system (Promega). To increase the amount of isolated DNA, an additional PCR with RAPD primers was performed, and the DNA was repurified using the gel electrophoresis and minicolumn procedure stated above.

The pGem-T vector system III kit (Promega) was used to clone the RAPD product, following the manufacturer's protocol. Colonies of white cells (as opposed to blue) indicated possible transformation. The candidate plasmid DNAs were isolated as described by Sambrook et al. (1989).

Two tests were conducted after plasmid isolation to confirm that the target RAPD fragment had been cloned. The plasmid plus insert fragment size was compared to the untransformed pGem-T plasmid size (in both circular and linear form) using agarose gel electrophoresis (Horejsi, 1998). The lack of plasmid insertion was determined by relative migration distance of DNA.

Secondly, products from PCRs with RAPD primers and the plasmid-insert vector as template DNA were compared to RAPD PCR products using cucumber template DNA (Horejsi, 1998). To test for the presence of plasmid PCR products and contamination, two controls were compared to each candidate clone. One control had no DNA and the second used pGem-T (without insert) as the template DNA. If the reamplified band was the same size as the RAPD band with no same-sized bands in the controls it was prepared for sequencing.

The cloned RAPD band was sequenced using the Silver Sequence System of Promega, following their protocol. Since the whole sequence was not needed, only a portion (25 to 200 bp) of each end was sequenced. The first and last 10 bases of each fragment were compared to the sequence of the original RAPD primers. If they were identical, it confirmed that a RAPD fragment had been cloned.

The SCAR primers evaluated in this study were designed for optimum PCR performance (for reviews of primer design see Sharrocks, 1994; Kocher and Wilson, 1991) using the primer select option of the DNASTAR 1.1.7 computer program (DNASTAR, Inc., Madison, Wis.) with the following constraints: 1) a primer length of 18 to 22 bases; 2) a maximum melting temperature of $51.0^{\circ}C$; 3) a minimum melting temperature of $38.1^{\circ}C$; 4) a unique 3' sequence of 7 bases; 5) a maximum dimer duplexing of two base pairs; 6) a maximum hairpin duplexing of two base pairs (ignore duplexing eight base pairs from 3' end), and; 7) a total target primer sequence and PCR products differ by no more than 60 base pairs. The resulting SCAR primer pairs are listed in Table 1. The SCAR marker nomenclature is according to Paran and Michelmore (1993); SCAR markers have the SC prefix placed before the name of their corresponding RAPD marker with its relative bp length, (e.g., marker SCH5₈₀₀ is the SCAR marker derived from RAPD H5₈₀₀).

The standard SCAR PCR protocol was identical to the standard RAPD protocol presented above, except for a two modifications. Because the SCAR PCR requires primer pairs (RAPDs have a single primer), the concentration of each SCAR primer was one-half (0.17 mM) that of the RAPD PCR. Likewise, the thermocycling protocol annealing step was changed to accommodate the longer SCAR primers: $94^{\circ}C/4$ min; 40 cycles of $94^{\circ}C/15$ s, $50^{\circ}C/15$ s, $59^{\circ}C$ ramp to $72^{\circ}C/75$ s; $72^{\circ}C/7$ min with subsequent cooling to $4^{\circ}C$.

EFFICIENCY OF RAPD TO SCAR CONVERSION. Four replications of each SCAR primer pair and their corresponding RAPD primers were subjected to the standard PCR protocols outlined above to compare the SCAR banding polymorphisms to their corresponding RAPD polymorphisms. The template DNA was from the cucumber genotypes known to have RAPD marker polymorphisms [G421, H19 (Serquen et al., 1997), WI 1983G, and Straight 8 (Horejsi, 1998)]. Efficiency of conversion was determined by comparison of SCAR and RAPD products for polymorphism detection. If the SCAR primers did not detect polymorphisms, they were evaluated to determine if higher annealing temperatures (55 , 60 , or $65^{\circ}C$) during PCR would allow for polymorphism detection.

SCAR AND RAPD PERFORMANCE UNDER VARIED PCR CONDITIONS. To test the effects of different $MgCl_2$ concentrations on SCAR and RAPD banding profiles, seven SCAR and RAPD primers (SCH5₈₀₀/H5₈₀₀, SCI4₁₁₀₀/I4₁₁₀₀, SCL19₉₄₀/L19₉₄₀, SCN6₈₀₀/N6₈₀₀, SCW7₁₁₅₀/

Table 1. Primer sequences and optimum annealing temperature of cucumber sequence characterized amplified regions (SCAR) markers and the corresponding random amplified polymorphic DNA (RAPD) markers from which they were derived.

SCAR					
			Optimum	RAPD	
	Primer sequence		annealing		Primer
Designation	(and number of nucleotides) ^z		temp (°C) ^y	Designation	sequence
SCB12 ₂₀₀	<u>TTGACGC</u> ACAAACAAAA (17)	<u>CCTTGACGC</u> ATCATTT (16)	63	B12 ₂₀₀	CCTTGACGCA
SCB12 ₃₅₀₀	TCATAAATCCCAATACCTCAAA (22)	TGGGAAATTAGTTAGCATCTCT (22)	61	B12 ₃₅₀₀	CCTTGACGCA
SCC1 ₄₀₀	<u>TCGAGCC</u> AGATGAGAAAAG (18)	CACGGAATAAGATACACAAAAGA (22)	53	C1 ₄₀₀	TTCGAGCCAG
SCC10 ₁₀₀₀	<u>TGGGTGC</u> ATTGAAACAGAGAC (21)	TGGTGAGGGGAGAGCATACTT (21)	58	C10 ₁₀₀₀	TGTCTGGGTG
SCF4 ₇₂₅	CTGCTTCCTGCATCAACAGA(20)	TTAATTAGTATTTCTCATAGTT (22)	52	F4 ₇₂₅	GGTGATCAGG
SCG14 ₈₀₀	TTAGAGCAAAAACTGGTGGTA(21)	TTAGGGAATGCAAGGAATG(19)	60	G14 ₈₀₀	GGATGAGACC
SCH5 ₈₀₀	<u>CTCGTCCC</u> TAAGAGTGTT (19)	TGAAAAATGAAGCAAAGGAT (20)	55	H5 ₈₀₀	AGTCGTCCCC
SCH13 ₁₁₀₀	CCCCCATGTTCTTCTTT (17)	TATTCATATTTGGCTCCAT (19)	58	H13 ₁₁₀₀	GACGCCACAC
SCI4 ₁₄₀₀	GAGCTTTATTCAACGCAACAT(23)	TATCTTTCTCACCTACCTTTATC(23)	64	I4 ₁₄₀₀	CCGCCTAGTC
SCI20 ₁₃₀₀	GCTACCTGTAAAGATGCT(18)	GGTTACCAAAGACAGATT(18)	51	I20 ₁₃₀₀	AAAGTGCGGG
SCJ5 ₁₀₀₀	<u>TCCATGGGG</u> GCTCTGTAC (18)	<u>CTCCATGGGG</u> TGTGCA (17)	62	J5 ₁₀₀₀	CTCCATGGGG
SCK7 ₅₀₀	CTCACGCAAAGCCCTCAGA(19)	TAGAAACTTCGAATAATCAGACAG(24)	57	K7 ₅₀₀	AGCGAGCAAG
SCK15 ₁₇₀₀	TCCTGCCAAATAAGAGA (17)	<u>TGCCAAT</u> CGATCCTAAAAC (19)	57	K15 ₁₇₀₀	CTCCTGCCAA
SCL18 ₆₀₀	ATTTGGTTATTATTTTATTTC(21)	<u>AACTCAC</u> CTCAAGATTTAGA(20)	57	L18 ₆₀₀	ACCACCCACC
SCL19 ₉₄₀	TCGAGGAACATCTTTACTT(19)	TTATTCTTATGTTGATCGCTTGTC(24)	64	L19 ₉₄₀	GAGTGGTGAC
SCN6 ₈₀₀	CTAGGGTTACAAAAATCCATAC(22)	TCCACGCCAAAATAATA(17)	59	N6 ₈₀₀	GAGACGCACA
SCN8 ₁₄₀₀	CAATAAAGAAGTCACCTCATCA (22)	<u>CCTCAGCT</u> CAACATTTACG (19)	58	N8 ₁₄₀₀	ACCTCAGCTC
SCP13 ₁₂₀₀	ACATTACCTAGTTTTATCG (19)	<u>GCCTCGA</u> CTACGGAAAAGA (18)	50	P13 ₁₂₀₀	GGAGTGCCCTC
SCP14 ₁₃₈₀	TGGATCGGGCAGGGCTTAGT(20)	GGGCGTGACCGAGACC(16)	69	P14 ₁₃₈₀	CCAGCCGAAC
SCR13 ₅₇₀	CACGCAAGAAAAGAAATGTAT(19)	TCCTTGTGAAATGCTAAT(19)	59	R13 ₅₇₀	GGACGACAAG
SCT2 ₉₀₀	ATAACACCATAGGCCAAGTCT (21)	<u>CTGTATG</u> TCAGATCAAAAACAAC (22)	62	T2 ₉₀₀	GGAGAGACTC
SCU15 ₁₉₀₀	ATCCAGCGCATTCTTTAG(18)	TTCGGCGGACTTGCTTTGGTGT(22)	67	U15 ₁₉₀₀	ACGGGCCAGT
SCW7 ₁₁₅₀	<u>GGACGTCA</u> ACTGAAAGGTAA (20)	<u>CTGGACGTCA</u> AATGTAGAGA (20)	61	W7 ₁₁₅₀	CTGGACGTCA
SCX15 ₁₁₀₀	AAGAATTTAGTTGTTGTTTGTGA(24)	GTGGGTGAAGGTGACGAT(18)	66	X15 ₁₁₀₀	CAGACAAGCC
SCY3 ₈₀₀	AGGGCCAAGCTGAACAAAA (19)	<u>CAGCCTG</u> CTCGGTATCCA (18)	58	Y3 ₈₀₀	ACAGCCTGCT
SCY5 ₇₇₅	CGTGAAGGCGTCGGCATAG(19)	ATCAATCATCCAGAGAATC(19)	61	Y5 ₇₇₅	ACAGCCTGCT
SCAA9 ₁₃₀₀	CGACCCGCCTCACTTAGC(18)	GTCTTACCGGCATTTTG(18)	69	AA9 ₁₃₀₀	AGATGGGCAG
SCAB14 ₇₈₀	AACTGATTTAAAGACAAGATT(21)	ATGGGGAGTGGGGAACAAAA(20)	57	AB14 ₇₈₀	AAGTGCGACC
SCAD12 ₇₀₀	AAGCTTCGCATTATTAGTGAT(22)	ATCCAAGCCGTCCATGAAAGTAT(23)	59	AD12 ₇₀₀	AAGAGGGCGT
SCAD14 ₈₀₀	CAGAAAACGAAAATGGTGTAAC(22)	ATTTGGATTTGGATATGCTGACT(23)	64	AD14 ₈₀₀	GAACGAGGGT
SCAF7 ₁₅₈₀	CGTACGGATTAAGCGAACCA(20)	AAGTGAATTTAAAGACCGAAAAA(24)	69	AF7 ₁₅₈₀	GGAAAGCGTC
SCAG17 ₈₂₅	TGATTTGGACACGACAGT(18)	TGAGGGGGAAAAAGAGA(17)	60	AG17 ₈₂₅	AGCGGAAGTG
SCA14 ₁₁₀₀	GAAGTCCGTGTCTATTATTGAT(22)	ATTACATTGTGGCAGTCTTTC(21)	65	A14 ₁₁₀₀	CTATCCTGCC
SCA110 ₃₅₀	AAATAGCACAAAGGTAGTTAGTT(23)	ATTGGGATAGTCTTGTTTCAT(20)	48	A110 ₃₅₀	TCGGGGCATC
SCAJ1 ₆₅₀	CTAGGGAGTTTGTGTTTGATGTG(22)	GATCCTCACGTGAAATGCTAT(21)	63	AJ1 ₆₅₀	ACGGGTCAGA
SCAJ18 ₁₀₀	TTTGTACGATGAAGGTTG(18)	AGATATCTCGAGCCATTGTTGA(22)	62	AJ18 ₁₀₀	GGCTAGGTGG
SCAK5 ₁₂₇₅	GGTATTGGTATGTTTTTCTATTTC(24)	GGTTTTACATCAGCCATCCT(20)	62	AK5 ₁₂₇₅	GATGGCAGTC
SCAM2 ₁₅₅₀	AGAAACGGCTACCACATCC(19)	ACGTAGTCAAGCGAGCAGA(19)	67	AM2 ₁₅₅₀	ACTTGACGGG
SCAN5 ₇₀₀	GGTATTGGTATGTTTTTCTATTTC(24)	GGTTTTACATCAGCCATCCT(20)	62	AN5 ₇₀₀	GGGTGCAGTT
SCAO7 ₁₁₀₀	TGCGAGCCAAATCCCATCT(19)	AGTGGAGTGGAGACGCAGAGA(21)	70	AO7 ₁₁₀₀	GATGCGACGG
SCAO12 ₁₃₇₀	CCTGTCACTTTGCTCCTAACTAA(24)	CTACGGATAAATCACCTGGACCTT(24)	68	AO12 ₁₃₇₀	TCCCGGTCTC
SCAR13 ₁₂₀₀	TTTAAATCTGGAACCTGT(18)	AAGCGCTGATACCAATTTG(18)	63	AR13 ₁₂₀₀	GGGTCCGGCTT
SCAS5 ₈₀₀	GACACTTTTGTGATAGCTTTCATT(24)	TCAACCAAAATTCACACTTAGA(22)	62	AS5 ₈₀₀	GTCACCTGCT
SCBC388 ₃₅₀	GCTCTATGGCGAAGTCAACCTCTC(24)	ACCAACCACCCAAGTAG(17)	53	BC388 ₃₅₀	CGGTCGCGTC
SCBC403 ₇₅₀	CGGATTTGACGGTAACT(17)	AAGGTCGAGGGATGTGC(17)	61	BC403 ₇₅₀	GGAAGGCTGT
SCBC469 ₄₀₀	TTGAGGGGAAAAATACATACC(20)	TGACAAACTTTAGGCTGACAT(21)	52	BC469 ₄₀₀	CTCCAGCAAA
SCBC519 ₁₁₀₀	AGATATAAGCGTTGTGAGGAT(21)	ATTATGATAGATTTCGTTTTTACC(23)	63	BC519 ₁₁₀₀	ACCGGACACT
SCBC526 ₉₀₀	GGGGGTGTGATTTAGTGC(18)	ATTACCTGGGATGCCTGTGC(20)	65	BC526 ₉₀₀	AACGGGCACC

²SCAR primer sequences, listed from 5' to 3', and number of nucleotides. The underscored sequence indicates the portion that is the same as the corresponding RAPD primer. Those SCAR primers were constructed from internal RAPD band sequences and contain no sequence in common to the RAPD primers of the corresponding RAPD marker.

³Optimum annealing temperature as calculated by the DNASTAR computer program (DNASTAR, Madison, Wis.).

W7₁₁₅₀, SCX15₁₁₀₀/X15₁₁₀₀, and SCY3₈₀₀/Y3₈₀₀) were evaluated at six MgCl₂ concentrations (13, 6, 3, 2, 1, and 0 mM) with four replications. These markers were chosen because the SCAR marker PCR products had the same mobility and allelic polymorphism as the original RAPD.

The effects of template DNA on the performance of each marker type were evaluated by examining 1) simulated interpopulation contamination (representing contrasting marker alleles) (0%, 1%, 2%, 3%, 5%, 10%, 90%, 95%, 97%, 98%, 99%, and 100%); 2) varying DNA concentrations (13, 6, 3, 2, 1, and 0 mM); and 3) polymorphisms among inbred lines, their progeny and diverse genetic backgrounds. Experiments that simulated contamination levels of one individual with tissue from a second individual were performed using mixtures of template DNA from the genotypes G421 (band presence) and H-19 (band absence) which possess RAPD polymorphisms (Serquen et al., 1997). Experiments studying the effect of varying DNA concentrations on PCR performance used DNA from G421 and H-19 with 10 polymorphic primers (SCB12₃₅₀₀, SCH5₈₀₀, SCI4₁₁₀₀, SCL19₉₄₀, SCN6₈₀₀, SCW7₁₁₅₀, SCX15₁₁₀₀, SCY3₈₀₀, SCAJ18₁₀₀, and SCBC388₃₅₀). DNA from cucumber lines (SMR18, GY4a, WI 1983G, WI 2757, Zudm1, and 'Straight 8') and 21 F₂ plants (from a Zudm1 X Straight 8 mating) were used as templates to compare the performance of two SCAR and RAPD markers (SCB12₃₅₀₀/B12₃₅₀₀ and SCX15₁₁₀₀/X15₁₁₀₀) from different sources (i.e., different genetic backgrounds) and in segregating progeny in two repetitions. Treatments in the interpopulation contamination, DNA concentration and polymorphism experiments were replicated two, four, and four times, respectively. All germplasm used in these experiments was received from the USDA Agricultural Research Cucumber Project, Madison, Wis.

Two sources of Taq DNA polymerase (Promega, lot# 6559611 and Pharmacia, Piscataway, N.J., lot# 6110798011) were evaluated for uniformity in generating SCAR or RAPD markers. Taq polymerase treatments (sources) were tested with four RAPD (B12₃₅₀₀, H5₈₀₀, W7₁₁₅₀, and Y3₈₀₀) and four SCAR (SCB12₃₅₀₀, SCH5₈₀₀, SCW7₁₁₅₀, and SCY3₈₀₀) markers in four replications. Three genotypes (H-19, G421, and WI 2757) were used for template DNA.

Two different thermocycling machines [the GeneAmp PCR System 9600 (Perkin Elmer) and the PTC-100-96 (MJ Research, Watertown, Mass.)] were used to compare potential differences in RAPD and SCAR tolerance. The GeneAmp PCR System 9600 used polyethylene reaction tubes. The PTC-100-96 uses trays made of polyvinylchloride. This plastic is known to bind proteins, including Taq polymerase (Griffin and Griffin, 1994). Therefore, a blocking protein of bovine serum albumin (Promega) was added (0.1 µg·µL⁻¹) to each PCR (in both machines) to competitively bind to the plastic.

ANALYSIS OF PCR PRODUCTS. To compare the amount of DNA produced and evaluate the relative ease of scoring of SCAR and RAPD markers, the DNA band intensity (fluorescence) was compared using PCR methodologies described above. The relative intensity (DNA content) of each band was estimated by visually comparing it to a standard of *Eco*RI plus *Hind*III digested lambda phage DNA, which displayed bands ranging from 1 to 130 ng of DNA. Comparisons between markers were made by examination of SCAR and RAPD PCR products on the same agarose gel with each replication. The Student's *t* test was used to determine the statistical significance of these comparisons.

Results and Discussion

EFFICIENCY OF RAPD TO SCAR CONVERSION. RAPD to SCAR marker conversion appears technically straight forward (Paran and

Michelmore, 1993). However, conversion of cucumber RAPD to SCAR markers was not universally successful. Although 62 (83%) of the 75 RAPDs were successfully cloned, only 48 (64%) RAPD markers were successfully converted to SCAR markers. The number of potential SCAR markers decreased at each step of the conversion process. Of the 75 RAPD bands originally extracted, 63 were successfully ligated into the pGEM-T plasmid, 62 were transformed, 54 sequencing templates were produced, and 48 were successfully sequenced. In the interest of time and resource efficiency, a maximum of five additional attempts were made to advance each RAPD fragment through each step of the conversion process. If such attempts did not advance a particular RAPD fragment to the following step of the conversion process, that RAPD was deleted from further consideration for SCAR conversion. The RAPD to SCAR conversion process is complex and requires strict control of experimental conditions. Thus, the efficiency of the conversion process might be improved by technical modification of critical steps in the conversion methodology.

Of the 48 SCAR primer pairs produced (Table 1), only 11 (15%) reproduced the polymorphism observed in the original RAPD PCR product (Table 2). This loss of polymorphism upon conversion was also observed by McDermott et al. (1994), Naqvi and Chattoo (1996), Ohmori et al. (1996), Paran and Michelmore (1993) and Witsenboer et al., (1995). No relationship was observed between PCR fragment length of a RAPD primer product and its successful conversion to a SCAR marker [i.e., both long (3500 bp) and short (100 bp) fragments were convertible].

The relative mobility (i.e., DNA fragment length) of the polymorphic DNA bands from RAPD markers was the same as their corresponding SCAR bands. The observed RAPD marker polymorphisms between G421 and H19 (Serquen et al., 1997) were also observed with 10 (23% of the 48 SCAR primer pairs produced) SCAR markers producing a single PCR product (SCB12₃₅₀₀, SCH5₈₀₀, SCH13₁₁₀₀, SCW7₁₁₅₀, SCY3₈₀₀, SCI4₁₁₀₀, SCL19₉₄₀, SCN6₈₀₀, SCAJ18₁₀₀, and SCBC388₃₅₀). Similarly, of the four RAPD marker polymorphisms observed between WI 1983G and Straight 8, only SCAR SCX15₁₁₀₀ detected a difference.

Theoretically, a SCAR primer pair should amplify the unique band cloned from the corresponding RAPD (Paran and Michelmore, 1993). Since SCAR primers are longer and thus more specific than their RAPD primer counterparts, they should yield fewer PCR products; ideally only one. However, if the SCAR primers have either duplicated sequences (i.e., segments with ubiquitous sequences) or sequences closely resembling multiple genomic regions, the PCR may yield multiple bands. A single band from a SCAR PCR with multiple bands can still give useful genetic information, similar to marker loci from a RAPD PCR. In most cases, the RAPD to SCAR conversion process resulted in fewer amplification bands in PCRs with SCAR primers compared to the RAPD primers from which they were derived (Table 2). Twenty-five SCAR primer-pairs (52%) yielded a single band, indicating the likelihood of a single PCR product (Table 2).

Amplification using 20 (42% of the 48 SCAR primer pairs produced) SCAR primer pairs yielded multiple bands (Table 2). Apparently, these primers do not possess unique or highly specific binding sites. For instance, two PCR products were produced by SCC10₁₀₀₀ while its corresponding RAPD produced only one. Likewise, SCAR primer-pairs SCB12₃₅₀₀, SCC10₁₀₀₀, SCH13₁₁₀₀, SCN6₈₀₀, SCW7₁₁₅₀, SCY3₈₀₀, SCAJ18₁₀₀, and SCBC388₃₅₀ produced several bands, however, only one band reflected the same polymorphism as its corresponding RAPD (i.e., absence or presence depending on genotype). If all additional bands were ignored and the polymorphic band (with the same mobility as the RAPD) was heritable, variation

Table 2. Comparison of polymerase chain reaction (PCR) banding patterns between cucumber sequence characterized amplified regions (SCAR) and corresponding random amplified polymorphic DNA (RAPD) markers.

SCAR primers ^z	Bands (no.) ^y	RAPD primer	Bands (no.) ^x	SCAR polymorphism detection ^w
SCB12 ₂₀₀	1	B12 ₂₀₀	4	No
SCB12 ₃₅₀₀	2	B12 ₃₅₀₀	4	Yes
SCC1 ₄₀₀	1	C1 ₄₀₀	5	No
SCC10 ₁₀₀₀	2	C10 ₁₀₀₀	1	No
SCF4 ₇₂₅	1	F4 ₇₂₅	6	No
SCG14 ₈₀₀	1	G14 ₈₀₀	12	No
SCH5 ₈₀₀	1	H5 ₈₀₀	6	Yes
SCH13 ₁₁₀₀	3	H13 ₁₁₀₀	7	Yes
SCI4 ₁₁₀₀	1	I4 ₁₁₀₀	10	Yes
SCI20 ₁₃₀₀	2	I20 ₁₃₀₀	8	No
SCJ5 ₁₀₀₀	1	J5 ₁₀₀₀	5	No
SCK7 ₅₀₀	1	K7 ₅₀₀	7	No
SCK15 ₁₇₀₀	1	K15 ₁₇₀₀	6	No
SCL18 ₆₀₀	1	L18 ₆₀₀	13	No
SCL19 ₉₄₀	1	L19 ₉₄₀	7	Yes
SCN6 ₈₀₀	2	N6 ₈₀₀	8	Yes
SCN8 ₁₄₀₀	1	N8 ₁₄₀₀	7	No
SCP13 ₁₂₀₀	1	P13 ₁₂₀₀	4	No
SCP14 ₁₃₈₀	3	P14 ₁₃₈₀	5	No
SCR13 ₅₇₀	2	R13 ₅₇₀	12	No
SCT2 ₉₀₀	1	T2 ₉₀₀	6	No
SCU15 ₁₉₀₀	2	U15 ₁₉₀₀	9	No
SCW7 ₁₁₅₀	3	W7 ₁₁₅₀	7	Yes
SCX15 ₁₁₀₀	1	X15 ₁₁₀₀	10	Yes
SCY3 ₈₀₀	5	Y3 ₈₀₀	8	Yes
SCY5 ₇₇₅	3	Y5 ₇₇₅	7	No
SCAA9 ₁₃₀₀	7	AA9 ₁₃₀₀	10	No
SCAB14 ₇₈₀	4	AB14 ₇₈₀	8	No
SCAD12 ₇₀₀	1	AD12 ₇₀₀	9	No
SCAD14 ₈₀₀	0	AD14 ₈₀₀	6	No
SCAF7 ₁₅₈₀	7	AF7 ₁₅₈₀	10	No
SCAG17 ₈₂₅	4	AG17 ₈₂₅	5	No
SCAI4 ₁₄₀₀	1	A14 ₁₄₀₀	3	No
SCAI10 ₃₅₀	1	AI10 ₃₅₀	11	No
SCAJ1 ₆₅₀	1	AJ1 ₆₅₀	7	No
SCAJ18 ₁₀₀	2	AJ18 ₁₀₀	7	Yes
SCAK5 ₁₂₇₅	1	AK5 ₁₂₇₅	5	No
SCAM2 ₁₅₅₀	3	AM2 ₁₅₅₀	5	No
SCAN5 ₇₀₀	1	AN5 ₇₀₀	4	No
SCAO7 ₁₁₀₀	0	AO7 ₁₁₀₀	4	No
SCAO12 ₁₃₇₀	1	AO12 ₁₃₇₀	6	No
SCAR13 ₁₂₀₀	2	AR13 ₁₂₀₀	8	No
SCAS5 ₈₀₀	0	AS5 ₈₀₀	4	No
SCBC388 ₃₅₀	5	BC388 ₃₅₀	8	Yes
SCBC403 ₇₅₀	3	BC403 ₇₅₀	6	No
SCBC469 ₄₀₀	1	BC469 ₄₀₀	3	No
SCBC519 ₁₁₀₀	1	BC519 ₁₁₀₀	3	No
SCBC526 ₉₀₀	1	BC526 ₉₀₀	7	No

^zMarker designation with band size in base pairs (subscript).

^yNumber of bands produced from PCR with SCAR primer pairs, including the critical polymorphic band.

^xNumber of bands produced from PCR with RAPD primer pairs, including the critical polymorphic band(s).

^wSCAR polymorphism detection agreement with their corresponding RAPD polymorphism.

^vThe SCN6₈₀₀ primer pair produced two polymorphic bands (SCN6₈₀₀ and SCN6₆₀₀). SCN6₈₀₀ was in agreement for polymorphism detection with the corresponding RAPD band (N6₈₀₀). Even though the SCN6₆₀₀ was polymorphic, the RAPD N6₆₀₀ was monomorphic.

^uThe SCY6₈₀₀ primer pair produced two polymorphic bands (SCY6₈₀₀ and SCY6₆₀₀). Both bands were in agreement for polymorphism detection with the corresponding RAPD bands (Y6₈₀₀ and Y6₆₀₀).

Fig. 1. Mean DNA content of polymorphic bands from polymerase chain reaction amplification with five randomly amplified polymorphic DNA (RAPD) primers (B12, H5, H13, Y3, Y3 and W7) and their derived sequence characterized amplified region (SCAR) markers (SCB12₃₅₀₀, SCH5₈₀₀, SCH13₁₁₀₀, SCY3₆₀₀, SCY3₈₀₀, and SCW7₁₁₅₀). **Significant difference between SCAR and RAPD values based on *t* test at $P \leq 0.05$ or 0.01, respectively.

in such SCAR markers could be utilized in genetic studies.

Two SCAR primer pairs (SCN6₈₀₀ and SCY3₈₀₀) produced additional polymorphic PCR products which were either not clearly evident or not often detected in RAPD PCRs of G421 and H-19. These additional polymorphic bands were designated as SCN6₆₀₀ and SCY3₆₀₀, respectively. The 600-bp band was observed in the RAPD banding pattern of Y3, but was often too faint to score, and therefore was not mapped by Serquen et al. (1997). Likewise, a 600-bp band was also observed in the N6 RAPD banding profile, but was not polymorphic between G421 and H-19. In these two cases, the increase in scorable polymorphic bands are examples of the potential increased effectiveness of SCAR markers over their corresponding RAPD markers.

The target polymorphism present in the RAPD was not observed in the PCR products of 37 (77%) of the 48 converted SCAR primer pairs (Table 2). While no DNA amplification (i.e., no bands detected) was observed for three SCAR primer pairs (SCA14₈₀₀, SCA07₁₁₀₀, and SCAS5₈₀₀), the polymorphism loss in the remaining SCAR primer pairs was due to the amplification of the target PCR product in all genotypes. Increasing the annealing temperature during PCR to 55, 60 or 65 °C allowed only one (SCAA9₁₃₀₀; 60 °C) of these 37 SCAR primer pair constructs to detect the target polymorphism observed in its corresponding RAPD.

RAPD polymorphisms can reflect point mutations in the primer annealing sites or in length differences between primer annealing sites due to insertion or deletion mutations (Williams et al, 1990). The loss of polymorphism during RAPD to SCAR conversions suggests that these RAPD polymorphisms were caused by site mutations at the primer binding site. The SCAR primer construct may mitigate the difference(s) in the nucleotide sequence responsible for the observed difference (mutational event), and thus may anneal to areas with common sequences to both genotypes resulting in a loss of polymorphism.

Even though the banding profiles of SCAR markers SCB12₃₅₀₀, SCH5₈₀₀, SCH13₁₁₀₀, SCW7₁₁₅₀, SCY3₈₀₀, and SCY3₆₀₀ were in agreement with their corresponding RAPD markers when G421 and H-19 were used as template DNA, occasionally marker inconsistencies were observed with DNA templates from these and other cucumber lines tested (GY14, SMR18, WI 1983G, and WI 2757) (data not presented). For instance, amplification of G421 and WI 2757 template DNA with SCAR SCW7₁₁₅₀ yielded PCR products

which were absent after PCR of their RAPD counterpart. In contrast, RAPD primer H5 amplified bands in PCR when employing G421, SMR18, and WI 1983G template DNA which were not amplified using its corresponding SCAR primer pair. Likewise, the amplification of G421 template DNA using RAPD primer Y3 produced a band (Y3₈₀₀), and its corresponding SCAR did not.

SCAR AND RAPD PERFORMANCE UNDER VARIED PCR CONDITIONS. To compare marker effectiveness, relative banding intensities of the marker types were compared. Under standard PCR conditions, SCAR primers on average produced significantly brighter bands ($P = 0.01$) than their corresponding RAPD primers (Fig. 1). Band intensities of SCAR markers SCH5₈₀₀, SCY3₆₀₀, and SCW7₁₁₅₀, were significantly brighter than that of their corresponding RAPD markers ($P \geq 0.05$). However, the intensity of bands produced by the RAPD H13₁₁₀₀ was significantly brighter ($P = 0.01$) than its corresponding SCAR, SCH13₁₁₀₀. The banding intensity of SCARs SCB12₃₅₀₀ and SCY3₈₀₀ was not significantly different from their corresponding RAPD markers. Greater band intensity may increase the ease and accuracy of scoring genotypes. Thus, some of the SCAR markers examined were superior to their RAPD counterparts, but others were not.

The response of SCAR and RAPD markers to various extreme PCR conditions was compared. Free Mg²⁺ is required for Taq polymerase activity and it also influences the annealing of primers to the template DNA (Kocher and Wilson, 1991). On average, the SCAR primer pairs (SCH5₈₀₀, SCH13₁₁₀₀, SCL19₉₄₀, SCN6₈₀₀, SCW7₁₁₅₀, SCX15₁₁₀₀, and SCY3₈₀₀) produced bands more dependably (significantly greater percentage, $P \geq 0.05$) than their corresponding RAPD primers over the MgCl₂ concentration range examined (Fig. 2A).

However, at the standard PCR (Staub et al., 1996b) concentration (3 mM MgCl₂), no difference in banding frequency (percentage of bands recorded) was detected between SCAR and corresponding RAPD markers. Staub et al. (1996b) reported that RAPD banding patterns in cucumber were detrimentally affected by low Mg²⁺ concentration (≤ 2.0 mM) in the PCR.

Our data indicate that the stable SCAR markers examined were less inhibited by

Fig. 2. Percentage of polymerase chain reactions (PCRs) producing randomly amplified polymorphic DNA (RAPD) or sequence characterized amplified region (SCAR) DNA bands at varying MgCl₂ concentrations (A) and DNA template concentrations (B) using seven SCAR markers (SCH5₈₀₀, SCH13₁₁₀₀, SCL19₉₄₀, SCN6₈₀₀, SCW7₁₁₅₀, SCX15₁₁₀₀, and SCY3₈₀₀) and their corresponding RAPD markers (H5₈₀₀, I4₁₁₀₀, L19₉₄₀, N6₈₀₀, W7₁₁₅₀, X15₁₁₀₀, and Y3₈₀₀) in the PCR. **Significant difference between SCAR and RAPD values based on *t* test at $P \leq 0.05$ or 0.01, respectively.

low and high (≥ 6 mM) Mg^{+2} concentrations than their RAPD marker counterparts.

Theoretically, the PCR should amplify sequences that are in low copy number (Mullis et al., 1986). Seven SCAR and RAPD markers were tested for sensitivity to different DNA concentrations (line G421 and H-19) in dilution mixtures (0 to 100%). The mixture (contamination) experiment quantified the percentage of the target site-present template from one genotype was detectable in DNA dilutions from a nonsite-present template genotype. SCAR markers SCB12₃₅₀₀, SCL19₉₄₀, and SCW7₁₁₅₀ were more sensitive to site-present template DNA (2%, 1%, and 2%, respectively) than the corresponding RAPD markers (B12₃₅₀₀, L19₉₄₀, and W7₁₁₅₀ at 5%, 5%, and $>10\%$, respectively). However, the SCAR markers SCAJ18₁₀₀ and SCBC388₃₅₀ needed a greater percentage of band-producing DNA ($>10\%$ and $>10\%$, respectively) than the RAPDs AJ18₁₀₀ and BC388₃₅₀ needed (5% and 2%, respectively). No difference was detected between the SCARs SCH5₈₀₀ (1%) and SC14₁₁₀₀ (2%) and RAPDs H5₈₀₀ (1%) and I4₁₁₀₀ (2%). Since SCB12₃₅₀₀, SCL19₉₄₀, and SCW7₁₁₅₀ detected lower percentages of band-producing DNA than their RAPD counterparts, they may be more amenable to bulked DNA analysis. On the other hand, the avoidance of foreign DNA sources (e.g., cucurbits and other related species) becomes more important (i.e., selecting clean leaf tissue for DNA extraction) when using SCAR markers.

Sensitivity to a wide range of template DNA concentrations is important when accurate DNA concentration of samples is not known. The DNA quantification step during extraction is time consuming. Using the fluorometer and requiring two replications for accuracy, a person can process ≈ 10 samples per hour (data not presented). In high through-put genetic analysis, attempts may be made to reduce or eliminate the DNA quantification step. With such modifications, increased variability for template DNA content would be expected. Tests of primer sensitivity to different concentrations of pure template DNA in this study indicated that both marker types produced a lower frequency of bands as the amount of template DNA decreases (Fig. 2B). The SCAR markers examined produced bands less dependably (lower percentage) than their RAPD counterparts during PCRs with 1 and 2 mM template DNA. Thus, these sampled SCAR markers tended to be more influenced by low template DNA than their corresponding RAPDs.

For a marker to have utility in genetic analysis it must be reproducible and have a genetic basis. SCAR markers SCB12₃₅₀₀ and SCX15₁₁₀₀ detected polymorphisms between Zudm1 and Straight 8 and demonstrated complete agreement when compared to RAPD markers among 21 F_2 plants (data not presented). The combined results of the sensitivity analysis conducted by this study indicate that SCAR markers in cucumber can be effective for genetic analysis if their polymorphic nature is well defined (Fig. 3).

Comparisons of SCAR marker and RAPD marker response to different sources of Taq polymerase and thermocycling machines revealed no differences ($P = 0.05$). Others have reported that Taq polymerase source affects RAPD banding patterns (Sobral and Honeycutt, 1993; Staub et al., 1996b; Weeden et al., 1992). Our observations are also in agreement with those of Weeden et al. (1992) who found that differing thermocycling machines did not affect RAPD banding patterns.

The hypothesis presented by Paran and Michelmore (1993) that SCAR markers may be more reliable than RAPD markers is supported to some extent by our data (Fig. 3). The SCAR markers evaluated generally produced PCR products that were brighter than their corresponding RAPD marker bands. Such band intensity differences make these SCAR markers easier to score when compared to their RAPD counterpart.

SCAR markers may have two additional advantages that were not investigated by this study. SCAR markers may be multiplexed such that primers of several SCAR markers could be combined in the same PCR. This would increase the through-put potential of SCAR markers for genetic analysis. SCAR markers in some cases can be made codominant if the SCAR PCR products are digested with a restriction enzyme before electrophoresis (Paran and Michelmore, 1993). However, these advantages would likely not be realized for all SCAR since reaction dynamics of the PCR are variable and production of codominant markers is not predictable. In addition, substantial investment to clone and sequence RAPD bands for RAPD to SCAR conversions, and marker testing is essential. Therefore, decisions regarding implementation and deployment of SCAR technology must be weighed against its development costs and the potential loss of the original RAPD polymorphism.

Fig. 3. Ethidium bromide-stained gel (0.6% agarose) of polymerase chain reaction amplification products of randomly amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) primers and genomic DNA of cucumber lines. (A) Duplicates of reactions using RAPD Y3 (lanes 1–2 from line G421 and lanes 3–4 from line H-19), SCAR (lanes 7–8 from line G421 and lanes 9–10 from line H-19), controls [RAPD primer without template DNA (lanes 5–6) and SCAR primer without template DNA (11–12)], and a *EcoRI* plus *HindIII* digested lambda phage DNA at three concentrations (lane 13 = 31 ng, lane 14 = 92 ng, and lane 15 = 30 ng). The single and double arrows indicates the position of the 600 (single arrow) and 800 (double arrow) bp polymorphic SCAR marker band (SCY3₆₀₀ and SCY3₈₀₀). (B) Same lane arrangement for RAPD and SCAR products derived from decamer primer H5 but having only one lambda phage DNA preparation (lane 13 = 7.5 ng) and where the single arrow designates the position of the SCH5₈₀₀ primer product.

The intended application of markers is also a consideration when determining if SCAR construction is practical. Genetic distances (or similar measure) in germplasm diversity analysis are calculated based on marker loci differences (polymorphisms). Skroch and Nienhuis (1995) found that error rates from scoring RAPD banding patterns did not significantly affect their genetic distance analysis (sampling variance was greater than error variance). Therefore, the potential enhancement supplied by SCAR technology for diversity assessment may not be practical in a species like cucumber where polymorphism level and RAPD to SCAR conversion rate is low. Scoring errors during RAPD analysis may have a substantial impact on genetic analysis, since differences among a few marker loci can change the interpretation of magnitude of the genetic variation. For instance, genetic markers are being used for seed purity testing and protein-based markers (i.e., isozymes) are employed for plant variety protection (PVP) (Staub et al., 1996c). In seed purity analysis, even low levels of marker polymorphism ($\approx 5\%$) are interpreted as impurity (Weeden et al., 1992). PCR-based marker technologies have been proposed for PVP (Staub et al., 1996c). If these technologies are to be used for PVP and patent application, the examiner must be confident that the descriptors (alleles) are distinctive and reproducible (Staub et al., 1996c). Therefore, the cost of RAPD to SCAR conversion should be measured against the potential use of a SCAR marker system.

The application of SCAR markers may extend to genetic studies such as map construction where SCAR loci are equally spaced throughout the genome. RAPD markers converted to sequence tagged sites (STS) (Olson et al., 1989) or SCAR markers (Paran and Michelmore, 1993) could be used as genomic landmarks and provide for common reference points for interlaboratory research. Such landmark loci may be more beneficial for crop species evaluated by many research laboratories and seed companies, such as maize (*Zea mays* L.) and tomato (*Lycopersicon esculentum* L.), than for minor crops, such as oat (*Avena sativa* L.) and cucumber where few laboratories evaluate markers and funding is limited. Conditions that would foster their utilization are needed to support SCAR development. It is clear, however, that where a tight linkage exists between a marker and a gene controlling an economically important trait, SCAR construction may be worthwhile for marker-assisted selection even in a minor crop (McDermott et al., 1994; Naqvi and Chattoo, 1996; Ohmori et al., 1996; Paran and Michelmore, 1993; Witsenboer et al., 1995).

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