Development and Characterization of PCR Markers in Cucumber

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ABSTRACT. Highly polymorphic microsatellites or simple sequence repeat (SSR), along with sequence characterized amplified region (SCAR) and single nucleotide polymorphisms (SNP), markers are reliable, cost-effective, and amenable for large scale analyses. Molecular polymorphisms are relatively rare in cucumber (Cucumis sativus L.) (3% to 8%). Therefore, experiments were designed to develop SSR, SCAR and SNP markers, and optimize reaction conditions for PCR. A set of 110 SSR markers was constructed using a unique, strategically applied methodology that included the GeneTrapper (Life Technologies, Gaithersburg, Md.) kit to select plasmids harboring microsatellites. Of these markers, 58 (52%) contained dinucleotide repeats (CT, CA, TA), 21 (19%) possessed trinucleotide repeats (CTT, ATT, ACC, GCA), 3 (2.7%) contained tetranucleotide repeats (TGCG, TTA, TAA), 4 (3.6%) enclosed pentanucleotide repeat (ATTTT, GTTTTT, GGGTC, AGGCC), 3 (2.7%) contained hexanucleotide repeats (CCCCAA, TAAAAA, GCTGCC) and 21 possessed composite repeats. Four SCARs (L18-3 SCAR, AT1-2 SCAR, N6-A SCAR, and N6-B SCAR) and two PCR markers based on SNPs (L18-2H19 A and B) that are tightly linked to multiple lateral branching (i.e., a yield component) were also developed. The SNP markers were developed from otherwise monomorphic SCAR markers, producing genetically variable amplicons. The markers L18-3SCAR and AT1-2SCAR were codominant. A three-primer strategy was devised to develop a codominant SCAR from a sequence containing a transposable element, and a new codominant SCAR product was detected by annealing temperature gradient (ATG) PCR. The use of a marker among laboratories can be enhanced by methodological optimization of the PCR. The utility of the primers has been optimized by ATG-PCR to increase reliability and facilitate technology transfer. This array of markers substantially increases the pool of genetic markers available for genetic investigation in Cucumis.

Since the advent of the polymerase chain reaction (PCR) (Saiki et al., 1988), many techniques have been developed to detect DNA polymorphisms. Such polymorphisms have utility in plant genetics and breeding (Gupta and Varshney, 2000; Knapp 1998; Lee, 1995; Staub et al., 1996c). DNA fragment and sequence analysis has been used for genetic mapping experiments (Bradeen et al., 2001; Tanksley and Nelson, 1996), diversity analysis (Horejsi and Staub, 1999), and more recently marker-assisted selection in many crop species (Quarrie, 1996; Romagosa et al., 1999; Shen et al., 2001; Yousef and Juvik, 2001). For instance, isozymes, random amplified polymorphic DNA (RAPD) and restriction fragment polymorphisms (RFLP) have been used in cucumber (Cucumis sativus) for genetic mapping and diversity analysis (Dijkhuizen et al., 1996; Horejsi et al., 2000; Kennard et al., 1994; Meglic and Staub, 1996a; Meglic and Staub, 1996b; Serquen et al., 1997). The use of these markers has been limited due to the narrow genetic base (3% to 8% on a per band basis) of cucumber (Horejsi and Staub, 1999; Kner et al., 1989). A higher level of polymorphism has been documented in C. sativus, and in Cucurbita and Citrullus species of the Cucurbitaceae using simple sequence repeat (SSR) (Katriz et al., 1996). Four of the seven SSRs used detected polymorphisms among the 11 cucumber genotypes examined which allowed for the calculation of gene diversity values ranging between 0.18 and 0.64 (Katriz et al., 1996). More recently, 20 polymorphic SSR's were developed and tested in cucumber to more critically determine their potential value for genetic analysis. Polymorphisms were detected at 12 of the 20 SSR loci (60%) having two to five alleles at each SSR locus examined (Danin Poleg et al., 2000; Danin Poleg et al., 2001).

A moderately large molecular marker database now exists for cucumber that includes isozymes (Meglic and Staub, 1996b), RFLP (Dijkhuizen et al., 1996), RAPD (Horejsi and Staub, 1999), amplified fragment length polymorphisms (AFLP) (Bradeen et al., 2001), SSR (Danin Poleg et al., 2000), and sequence characterized amplified regions (SCAR) (Horejsi et al., 1999) markers. The application of these markers for diversity assessment has allowed for the partitioning of commercial germplasm into market classes, and the grouping of accessions in the U.S. National Plant Germplasm System into geographic proximity and country of origin. Their limitations for use in legal applications stem from their paucity, degree of consistency, and cost (Staub, 1999; Staub et al., 1996b; Staub and Meglic, 1993).

The characterization of additional polymorphic SSR, single nucleotide polymorphisms (SNP), and SCAR markers would allow increased precision in genetic similarity estimation among cucumber cultivars as well as in the mapping of qualitative and quantitative traits. Despite their high development costs, microsatellites have played an important role in genetic studies of higher eukaryotes, such as sweetpotato (Ipomoea batatas L. Lam.) (Buteler et al., 1999), cassava (Manihot esculenta L.) (Chavarriagaquira et al., 1998), peach (Prunus persica L.) (Cipriani et al., 1999), barley (Hordeum vulgare L.) (Donini et al., 1998), and beans (Phaseolus vulgaris L.) (Yu et al., 1999). Where SSR size polymorphisms can be resolved by high throughput and inexpensive technologies (e.g., agarose gel analysis), their codominant nature, and relatively high polymorphism rate make them amenable for many types genomic analyses (Donini et al., 1998;
Gupta et al., 1999). Likewise, the conversion of a RAPD marker to SCAR or SNP markers can enhance the marker’s repeatability (Michelmore et al., 1991; Staub et al., 1996a).

The initial development of microsatellite markers requires the characterization of sequences flanking the repeat motif followed by the design of flanking PCR primers. Several methods have been devised for the initial characterization of the flanking sequences (Gupta and Varshney, 2000) including the use of a published gene sequence (Litt and Luty, 1989), database searches (Danin Poleg et al., 2000; Danin Poleg et al., 2001; Katzir et al., 1996), the construction of genomic or cDNA libraries with subsequent screening with labeled oligonucleotide probes (Bryan et al., 1997; Dayanandan et al., 1998; Huang et al., 1998; Roder et al., 1998), the construction of microsatellite-enriched libraries followed by PCR screening (Fischer and Bachmann, 1998), hybridization of biotinylated oligonucleotides to single stranded M13 libraries (Paetkau, 1999), and the use of bacterial artificial chromosome (BAC) libraries to target specific genomic regions (Cregan et al., 1999). The development of SSR marker technology for cucumber is a necessary step to increase map saturation for a more complete analysis of its genome and the application of this information for its improvement. A method for the discovery of microsatellites and their flanking sequences is described herein along with the identification of 2 SNP markers and the development of 110 microsatellite and 4 SCAR markers and their optimization in the PCR.

Materials and Methods

SSR marker development

The development of SSR markers (Fig. 1) involved the construction of a cucumber DNA genomic library, and subsequent capture of library inserts aided by GeneTrapper technology, followed by the sequencing of captured products (Fazio and Staub, 2000; Fazio, 2001). Sequence information was then used to develop primer pairs, which flanked target SSR regions.

DNA extraction, restriction and size fractionation. Genomic DNA from cucumber line GY7 (released by the University of Wisconsin, 1996, R.L. Lower; evaluated as experimental line G421) was extracted from young leaves (1 to 15 cm²) and apical meristems according to a nuclear DNA extraction protocol (Sambrook et al., 1989). Extraction products were treated with RNase ONE (Promega, Madison, Wis.). Recovered DNA was quantified using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). In separate reactions, 100 mg of DNA was restricted using EcoR I* restriction enzyme (Promega) and Acs I (Roche, Indianapolis, Ind.), which recognizes the sequence (A or G)/AATT(T or C), and creates compatible ends with EcoR I restriction site. The star activity in EcoR I was initiated by adding 10% DMSO to the restriction buffer (Anderson and McDonald, 1993; Robinson and Sligar, 1995). This activity generated additional restriction fragments ranging 200 to 1300 bp as opposed to EcoR I normal activity.

Since the then available sequencing techniques could only resolve ≈800 bp, fragment sizes between 200 to 1300 bp were needed to avoid unnecessary subcloning and to simplify the sequencing process. Restricted DNA was size-fractionated using a low pressure, 75-cm-long, 16-mm-diameter, chromatography column (Fisher Scientific, Pittsburg, Pa.) filled with Sephacryl S-500 (Pharmacia, Peapack, N.J.). The column was packed according to manufacturer’s specifications for Sephacryl S-500 and equilibrated with 200 mL elution buffer (0.1 m Tris-HCL pH 8.0, 0.15 m NaCl, 0.001 m EDTA) at 0.3 mL·min⁻¹ before fractionation. Both samples (EcoR I* and Acs I) of restricted DNA were fractionated (0.3 mL·min⁻¹) separately, and eluted DNA was collected in 75 2-mL aliquots. Eluted DNA was precipitated, washed and resuspended in TE. Fragments from eluted aliquots of both restrictions were then sized by agarose gel electrophoresis.

Fig. 1. Methodology for the development of cucumber simple sequence repeat (SSR) markers using the GeneTrapper kit (Life Technologies, Gaithersburg, Md.).
and simultaneously compared to the nonfractionated digestion products from each restriction. Fractions between 200 to 1200 bp were chosen to create a small insert genomic library, and then they were combined for ligation to a vector. These size fractions were chosen because of the increased difficulty of sequencing larger size inserts.

**Library construction, mass excision, and plasmid DNA extraction.** Fractionated DNA was ligated to Lambda Ziplox EcoRI arms (Life Technologies, Gaithersburg, Md.), and then it was packaged in a lambda vector with Gigapack III Gold packaging extract (Stratagene, La Jolla, Calif.). The resulting library was titered and packaging efficiency was determined by blue-white colony screening following the manufacturer’s specifications.

Primary Ziplox libraries were mass excised *in vivo* into the plasmid vector, pZL1 (Life Technologies) in the strain of *E. coli* DH10B Zip (Life Technologies) as per manufacturer’s recommendation. The mass excision of the lambda vector into a plasmid and the expansion of the plasmid library were performed simultaneously in a semi-solid medium according to the manufacturer to minimize representational biases (some cell lines dividing faster than others) that can occur during colony expansion in liquid media. The growth media was prepared by adding 1.35 g of SeaPrep agarose (FMC Bioproducts, Rockland, Maine) to 500 mL of 2 × LB in an autoclavable bottle using a stir plate to avoid the formation of agarose clumps. The bottle was autoclaved for 30 min and cooled in a water bath until the media reached 37 °C. Ampicillin (Fisher) was then added to the media (150 mg·L⁻¹) and 5 × 10⁹ primary transformants from the ZipLox libraries were added to the bottle while being stirred. The bottle was subsequently placed in a 0 °C water bath for 1 h to solidify the media. The culture was incubated undisturbed at 30 °C for 45 h, and then centrifuged at 3000 g, for 20 min. The supernatant was discarded, and the recovered cells were resuspended in 100 mL of 2× LB Glycerol (12.5%). Subsequently, 100 mL of LB media were inoculated with 2.5 × 10⁶ cells resulting from the mass excision, and the mixture was then cultured at 30 °C until the culture reached an OD of 0.8. Plasmid DNA to be used in the GeneTrapper protocol was extracted by with the plasmid Maxi-Prep Kit procedure (Qiagen, Valencia, Calif.).

**Capture of plasmids containing microsatellites.** In preparation for the GeneTrapper (Life Technologies) reaction, five oligonucleotides (20 to 30 bp) homologous for common microsatellite motifs [(CT)₁₂, (TG)₁₂, (CTT)₉, (TCC)₉, (ATT)₉] were modified at the 3' end by the addition of biotin (biotin-14-dCTP) with a terminal transferase enzyme provided with the Gene II protein and Exonuclease III to obtain single-stranded plasmid DNA. The derived from the cucumber DNA library survived the selection media challenge.

6) Single colonies were picked, named sequentially, and used to inoculate 3 mL of LB Ampicillin (100 µg·mL⁻¹). The inoculated media was incubated at 37 °C overnight, and then plasmid DNA were extracted using the QIAPREP miniprep kit (Qiagen) according to the manufacturer’s protocol. Plasmid DNA was amplified by PCR [3 mM MgCl₂, 0.2 mM dNTPs, 15 to 20 ng of DNA, 0.4 mM of each primer, Taq polymerase (3 units) and commercial 10× buffer (Promega)] containing the capture oligonucleotide along with the standard forward or reverse M13 primer to confirm the presence of a microsatellite in the plasmid.

7) Plasmid DNA (Step 5) (4 µL at 200 ng·mL⁻¹) from positive clones was added to a 16-µL sequencing reaction mixture containing 4 µL of 2.5× reaction buffer [5× is 400 mM Tris pH 9.0, 10 mM MgCl₂], 4 µL of BigDye enzyme mix (Applied Biosystems, Foster City, Calif.), 1 mL of M13 forward or reverse primer (20 pmol·µL⁻¹), and 7 µL of distilled water. Sequencing reactions were run on a thermal cycler (9700;
DNA in PCR to identify single nucleotide polymorphisms and/or polymorphisms caused by insertion/deletions (Indel) within SCAR markers (agarose analysis) monomorphic between G-421 and H-19. These markers were selected because of their potential usefulness in current mapping projects.

Two putative loci (L-18-2-SCAR, and N6-SCAR) were amplified using SCAR primers in both of the mapping inbred lines and two loci (RAPD L-18-3, AT1-2) were amplified with RAPD primers L-18 and AT1 (Operon Technologies, Alameda, Calif.), L-18-2-SCAR, was monomorphic between G-421 and H19 lines and N6-SCAR amplified bands of different sizes in H-19 and G-421 suggesting the possibility of a codominant genetic nature. Further analysis revealed that N6-SCAR marker under standard PCR conditions failed to amplify the higher molecular weight band in the F1 hybrid as well as in the heterozygotes in an F2 population thus making this potentially codominant marker behave as a dominant one. Because of these attributes, experiments were designed to improve the performance of those markers.

Amplicons were separated on a 1.6% agarose gel (Life Technologies) and stained with ethidium bromide (0.5 µg·mL−1). Banding patterns were visualized using a Dark Reader transilluminator, which does not use UV light, and therefore does not damage the DNA during visualization ultimately increasing transformation efficiency during subsequent cloning procedures. DNA bands were cut out of the gel and extracted using the Qiagen Gel Extraction Kit (Qiagen). The protocol from the pGEM-T Easy Vector System II cloning kit (Promega) was followed to ligate PCR products into the pGEM-T vector and transform competent JM109 E. coli cells with the ligated vector-insert. Plasmid DNA of colonies with positive insertional events identified by blue-white visual screening were extracted, and then sequenced using the same methods described in the previous section (Fig. 1, Step 7, microsatellite capture). Likewise, sequence analysis and primer design were performed using software and procedures previously described. However, in this case sequences of each marker band from line H-19 were compared to corresponding band sequences in G-421 to identify mismatch polymorphisms (SNPs) and insertion-deletion (Indel) polymorphisms. Sequence comparison information was then used strategically to design SNP and SCAR primers.

Optimization and customization of primer-pair-specific PCR conditions

An optimal PCR primer-pair design for amplification of specific DNA can be predicted by computer driven formulae and algorithms (Breslauer et al., 1986; Freier et al., 1986; Rychlik and Rhoads, 1989; Rychlik et al., 1990). Factors considered by these programs include 1) primer melting temperature (Tm), 2) product melting temperature, 3) primer sequence uniqueness with respect to the source sequence, 4) primer GC content, and, 5) 3’ end GC base anchor motif. However, such calculated properties often do not coincide with the information obtained from empirical assessment of primer-pair product performance obtained under different reaction conditions. Thus, PCR optimization of primer pairs designed to amplify SSRs, SNPs, and SCAR markers was performed by subjecting such primers to annealing temperature gradient PCR (ATG-PCR). Temperature gradient PCR amplifications were performed in a Eppendorf Master Cycler (Eppendorf, Hamburg, Germany) having a standard 12 column (1 to 12) × 8 rows (A to H) microtiter format providing row-dependent response information across rows (i.e., 12 temperatures in each column). The PCR reactions were performed in 15-µL volumes of PCR mix [3 mM MgCl2, 0.2 mM dNTPs, 15 to 20 ng of DNA, 0.4 mM of each primer, 2 units of Taq DNA polymerase and 10× commercial buffer (Promega)] overlayed with 10 µL of light weight mineral oil (Fisher). The ATG-PCR cycling conditions were conducted in split-well plates (Robbins Scientific, Sunnyvale, Calif.) as follows. A melting step (94 °C for 3 min), 40 PCR cycles (30 s at 94 °C, 1 min gradient annealing step 45 to 65 °C, and 90 s at 72 °C), and an elongation step (7 min at 72 °C).

Initially, DNA from H-19 and G-421 was used to detect locusspecific polymorphisms. A PCR master mix was initially prepared without DNA or primer, and then divided into two aliquots to which DNA was added. Each PCR master mix was then divided into eight aliquots to which primer pairs were added resulting in primer pair × DNA arrays having 12 temperature gradient replications. Split-well technology resulted in PCR reactions in each well containing identical primer pairs but different DNA (H-19 and G421) such that gradient temperature variations were minimized in each paired comparison. PCR reactions were resolved on 2% agarose gels (Life Technologies) with ethidium bromide staining (0.5 µg·mL−1), and visualized using a Dark Reader transilluminator coupled with a closed circuit digital (CCD) camera.

Results and Discussion

Development of PCR markers based on simple sequence nucleotide repeats

Digestion, size fractionation and plasmid library con-
Table 1. DNA sequences, optimal annealing temperature (°C), and expected product size (EPS) (bp) for 110 SSR and two SCAR markers characterized in cucumber (*Cucumis sativus* L.).

| No. | Marker | Sense primer 5’ to 3’ (top) | Antisense primer 5’ to 3’ (bottom) | SSR motif | EPS (bp) (top) | Temp (°C) (bottom) |
|-----|--------|-----------------------------|-------------------------------------|-----------|----------------|-------------------|
| 1   | CS-AT1SCAR | CACTCTTTTGAGGAGGGACATTGTG | AACCTCAATTTAATACAAAGAGATG | IN-DEL, SNP | 210 | 45–57 |
| 2   | CS-L18-3SCAR | CTTCTTCAATCTCTTCTTCTTCT | ATCATACAAATGAGATATTTTAC | IN-DEL | 327 | 45–57 |
| 3   | CSWACC01 | GATTACGGCACCCTTTTGAACGAG | TGTTTTGGCTCTTTTCCAATAGATGCCC | (ACC)5(A)8 | 305 | 57–65 |
| 4   | CSWACC02 | TGGCGGTGGGAACCTCCTTATGGTT | CCTCTTCAAGACTGTAGAGACGAT | (ACC)7 | 227 | 58–60 |
| 5   | CSWACC03 | ACAATAGGTCCTTTTTATGATAAT | TTTGGAACAGAAGGTGTCTCAGTAC | (GGT)5 | 279 | 53–65 |
| 6   | CSWATT01 | ATATGTTGATCTTAAACTAAGAGATG | TTATCCAAATACCATCAGGGATGAT | (TG)4(ATT)3 | 218 | 55–65 |
| 7   | CSWATT02 | CCAGTACAATAAACATCCGGAACG | ATCACCTTCTTTCAGTACGGGAT | (ATT)5 | 312 | 55–65 |
| 8   | CSWATT04 | TTTTGGGCATCTCCTACAGTTAGA | TTATCCTCGATCAGTGATGAG | (TAA)4TTA | 252 | 55–65 |
| 9   | CSWATT05 | CGGTGAAAGAAGAACATACAGA | TTTATGTAATAATTGTCTTAGTGT | (CTT)5(TAA)4 | 244 | 45–57 |
| 10  | CSWCOM03 | TTGTCATACAGGGCCCTCCGTG | ACTGTGTTGGCTGATACCTGCAT | (CCCAA)3 | 278 | 45–65 |
| 11  | CSWCOM04 | CATTACAACAAATCTCAGTTCG | GTAAAAATGGATAGACCCGATTT | ((AC)2(GC)2)(AC)4 | 291 | 45–50 |
| 12  | CSWCOM05 | CCACATTAACCCAAATACCCCCTCA | GACTGACCAAGAAGACCATACC | (TAAAAA)3(G)11 | 164 | 45–50 |
| 13  | CSWCOM06 | GAATCAAGTATACATGGAAATG | AGATTTAAAGGTATACACAT | (GAAA) motif | 275 | 45–62 |
| 14  | CSWCT01 | TTCTGATACAAGACGAGAG | GAACAAACAGGCTCCATTG | (CT)9(CT8) | 242 | 58–65 |
| 15  | CSWCT02B | TTCTGCATACCTCTCCTCT | CACACTTCTGCAGTGATG | (CT)21(TG)8 | 203 | 55–58 |
| 16  | CSWCT03 | TTCTCAGAAGCTGCACCT | CACTCTTGAGGGGGAAAA | (CT)16 | 390 | 48–52 |
| 17  | CSWCT04 | ACTATGGGTGTCCTCTCCT | GACCCCCAGGTTATTATT | (CT)6 | 556 | 48–54 |
| 18  | CSWCT05A | GTGTCGAAAGTAGCACCACCTCATAT | ATTCCTATAAAAATATAAGATGGTCG | (A)5(A)11 | 321 | 47–62 |
| 19  | CSWCT05B | ATACCAAGTCCTTCTTTTATAGG | ATTAAGGAGATAAGATGTGTG | (CT)14, (T)10 | 224 | 47–62 |
| 20  | CSWCT06A | TTTAAATCTCTTCTAACCC | TTGCTTCTGACATTTGAT | (CT)9 | 200 | 45–55 |
| 21  | CSWCT07A | GTACATCAATTGTTTCTAAATG | TTGCGGTGGGAAGGATATGAAATAG | (GT)TTT4 | 218 | 45–62 |
| 22  | CSWCT07B | CTCTCTCATTTATATCCTCCTCA | CCATATATCATCCTGATAGAAAAATTTG | (CT)10 | 196 | 48–60 |
| 23  | CSWCT07C | CAATTTTACACAGATGAAATAGTGG | GATTTTTATAGTTCACTCTCATAAC | (ATT)TTT3 AAATTT | 316 | 47–62 |
| 24  | CSWCT08 | ATTTCTTTAACCACACACA | ACCGTGTTGGGTAAGATG | (CT)11 | 594 | 65–68 |
| 25  | CSWCT09 | TATCTTTAAATGGTTTGC | ATAGCCCTTGGATATTGTT | (CT)10 | 273 | 45–55 |
| 26  | CSWCT10 | AGATCGGAATTGAAAAAG | AAGGGGCTTCTTTCTCA | (CT)9TTTTTCT | 183 | 55–60 |
| 27  | CSWCT11 | ATAGGCAATAGGCGTCTCT | CACTCTCAATGGGAGTTTTCG | (CT)9(A)13 | 226 | 45–50 |
| 28  | CSWCT12A | GAATTTCCGTCGCCCTTCGAGGC | GAGCCAAACCTCTGATGCGGAGTG | (GCTGGC)4(CT)12 | 297 | 61–65 |
| 29  | CSWCT12A | GAATTTCCGTCGCCCTTCGAGGC | ATGCCTAAGAGGATGATGAT | (GCTGGC)5 | 174 | 55–65 |
| No. | Marker | Sense primer 5’ to 3’ (top) | Antisense primer 5’ to 3’ (bottom) | SSR motif | EPS (bp) (top) | Temp (°C) (bottom) |
|-----|--------|-----------------------------|------------------------------------|-----------|---------------|------------------|
| 30  | CSWCT12B | TCAAATCATCTCATCTGTCAGTC     | TGAAGCAAAGATAGATGGGAATGG         | (CTT)2(CT)12 | 172           | 63–68            |
| 31  | CSWCT12C | TCAAATCATCTCATCTGTCAGTC     | TGAAGCAAAGATAGATGGGAATGG         | (CTT)2(CT)12(CTT)3 | 332           | 63–68            |
| 32  | CSWCT13B | TGGATCAACCAACTCA            | GAATAATGGGTCATTTT               | (CT)17(TG)13 | 290           | 45–58            |
| 33  | CSWCT13Balt | TGGATCAACCAACTCA       | GAATAATGGGTCATTTT               | (CT)17(TG)13 | 293           | 45–58            |
| 34  | CSWCT13C | AGAATCATTTCAACCA            | CATATGCGGATTCAC                | (GAA)motif | 278           | 45–62            |
| 35  | CSWCT14  | TCAAATGCGGATTCAC           | CATATGCGGATTCAC                | (GAA)motif | 226           | 58–62            |
| 36  | CSWCT15  | GAAATTCGTACCTGCGCAAGAGAGA  | GAATAATGGGTCATTTT               | (CTT)19(CA)6 | 193           | 55–62            |
| 37  | CSWCT16B | CTATATGCGGATTCAC           | CATATGCGGATTCAC                | (GAA)motif | 257           | 55–65            |
| 38  | CSWCT17  | TTGAATTTGCTCATCTG          | CATATGCGGATTCAC                | (GAA)motif | 286           | 45–48            |
| 39  | CSWCT18b | AAATAAATATACGTTGCGAT       | GAATAATGGGTCATTTT               | (CTT)19(CA)6 | 193           | 55–62            |
| 40  | CSWCT19  | AAATAAATATACGTTGCGAT       | GAATAATGGGTCATTTT               | (CTT)19(CA)6 | 193           | 55–62            |
| 41  | CSWCT19alt | AAATAAATATACGTTGCGAT    | GAATAATGGGTCATTTT               | (CTT)19(CA)6 | 193           | 55–62            |
| 42  | CSWCT20  | GAAATTTGATCCATAGATAGAT     | CATATGCGGATTCAC                | (GAA)motif | 226           | 58–62            |
| 43  | CSWCT22A | GGGATGATCGAAAGAAGGC        | TCTTGATCAACAGGACGAGTTAA         | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 44  | CSWCT23  | GAGAATATTCTGCCATATTCTC     | AAAAGATTAATTTTGACGGTAGAT        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 45  | CSWCT24A | AGGACATTGGGGAAGCTATAGTA    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 46  | CSWCT24B | ATCGCTTTATCGTGGGATATGTAC    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 47  | CSWCT24C | ATCGCTTTATCGTGGGATATGTAC    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 48  | CSWCT25  | GAATAAATATACGTTGCGAT       | GAATAAATATACGTTGCGAT           | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 49  | CSWCT26  | GCAATGTGCTGCGCAACATGAGA    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 50  | CSWCT27  | ATCGCTTTATCGTGGGATATGTAC    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 51  | CSWCT28  | GAATAAATATACGTTGCGAT       | GAATAAATATACGTTGCGAT           | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 52  | CSWCT29  | TGGACGAGTTGCTTGGTAAGCT     | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 53  | CSWCT30  | ATCGCTTTATCGTGGGATATGTAC    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 54  | CSWCT31  | GAATAAATATACGTTGCGAT       | GAATAAATATACGTTGCGAT           | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 55  | CSWCT32  | GCAATGTGCTGCGCAACATGAGA    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 56  | CSWCT33  | ATCGCTTTATCGTGGGATATGTAC    | ATCGCTTTATCGTGGGATATGTAC        | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 57  | CSWCTT02A | AGAATAACCCGCGGTCAAT        | GTAGGGTATATACAAAA              | (ATT)5(A)8(A)7 | 419           | 45–55            |
| 58  | CSWCTT02C | AGAATAACCCGCGGTCAAT        | GTAGGGTATATACAAAA              | (ATT)5(A)8(A)7 | 419           | 45–55            |
| No. | Marker       | Sense primer 5’ to 3’ (top)               | Antisense primer 5’ to 3’ (bottom)  | SSR motif                | EPS (bp) (top) | Temp (°C) (bottom) |
|-----|--------------|------------------------------------------|------------------------------------|--------------------------|----------------|-------------------|
| 59  | CSWCTT02D    | CATCCTTCAATTCATGGGAGGTGTG               | GAAATTGTTAAAAATGTACACATAA          | (T)6, (T)11, (CTT) motif | 246            | 45–60             |
| 60  | CSWCTT02D    | CATCCTTCAATTCATGGGAGGTGTG               | GAAATTGTTAAAAATGTACACATAA          | (T)6, (T)11, (CTT) motif | 246            | 45–60             |
| 61  | CSWCTT03     | GCATATCGATTTAGGTTCAAT                 | GGATGAGGCTGTTCTCTTA               | (CTT)4(T)11             | 184            | 49–52             |
| 62  | CSWCTT04     | GCGGAGATATGCCAATTTCAA                 | TCAAGCGGCACGGCAAT                 | (CTT)4(A)13             | 231            | 50–65             |
| 63  | CSWCTT05     | AGTATAGGATGATGAATGTGCGATT             | GAATTTGTTAAAAATGTGCCAAT           | (CTT)5                  | 424            | 45–50             |
| 64  | CSWCTT06     | TTTGAAAACTTTGATCCCAGTTTCTTC          | CAGAAAGGCATGTTATTATGCTGTA         | (GTT)2(CTT)5(ATT)2     | 423            | 52–65             |
| 65  | CSWCTT07     | TTTGTTGCAGGCAGCTGCTATAGGGT           | GAATTTCAATCAAGTGGAGCGATG          | (CTT)6                  | 275            | 54–65             |
| 66  | CSWCTT08     | GATAATAAGCTTGTGAGGATATG              | CTGGCTCTTGAATGAATTTAGTA           | (TAA)3(CTT)6            | 335            | 55–65             |
| 67  | CSWCTT09     | AAATTCTTCCCCATCTCACAATCT               | AAATTCTTCCCCATCTCACAATCT          | (CTT)5                  | 336            | 45–50             |
| 68  | CSWCTT10     | CTTCTACCCCAAAAACCCACCAATTC             | AGCCCTTCTTCAATCTCAGAAG             | (CTT)5                  | 371            | 52–55             |
| 69  | CSWCTT11     | ACAAGGGCGACAGCTGTCAC                  | TTCAGCTTTTTATCTATGATG             | (CT)6(CTT)4(CT)9        | 382            | 60–65             |
| 70  | CSWCTT12     | CTGTCCCTCTTGAAAAACGACATGTG            | ATGTACAGTTGAGCAATTTCTGCA          | (CTT)6                  | 227            | 61–65             |
| 71  | CSWCTT14     | AAAATATGAAAAGCCCATGACATG             | GATGCTAAATTTGGAGAATTCTAA          | (GAA)9(TA)4           | 263            | 45–52             |
| 72  | CSWGAAA01    | TTAGACCTTTTTTACTCTATGATG             | TTATTTTTTAAACAGATTITCA           | (CT)4                   | 218            | 45–55             |
| 73  | CSWGAAA02    | AGGCCATTGAGAAAATTTCTGATAAA           | AGCGGAGATTGGGACCGATTGGTAAT         | (CTT)8                  | 316            | 45–65             |
| 74  | CSWGAAAT01   | TGCCAGCTTGTGAGAGAGATG                 | GTGCTGACCTTTGACGCTTGTGAGGA         | (GAAT)5                | 276            | 45–65             |
| 75  | CSWGAT01     | ACCCGTGCTGATGCTCTC                   | CGGACCTTACGGGATGAA               | (CTT)4                  | 235            | 45–55             |
| 76  | CSWGATT01A   | ATTCACAGCITTTCTTCGAAAAAG            | CAGAATTCTTTGCTGTTGACTGTA          | (A)12(, GA)10          | 377            | 55–68             |
| 77  | CSWGATT01B   | TATCAGACGATTTTGCCACAGCCAB            | CAGAATTCTTTGCTGTTGACTGTA          | (GA)10                 | 181            | 65–68             |
| 78  | CSWGATT01C   | TATGGAACAGAAATATACATTGG               | CAGAATTCTTTGCTGTTGACTGTA          | (GA)10                 | 155            | 55–68             |
| 79  | CSWGATT01E   | GTGACATATGCTTTCTCTTC                 | GTGACATATGCTTTCTCTCTCTGCT         | (GGGT)3, (AGCC)3       | 235            | 55–68             |
| 80  | CSWGCA01     | AGTGAGATGGCAGCGCTATCTAT             | AGGATTTGACGTATGACGTT             | (GCA)8                 | 235            | 55–65             |
| 81  | CSWGTA01     | TGGATGATTGTTTCTTACAGA               | TTGTACCTTCCTCTTCTCTCTGCT         | (GTA)5, (GAAA) motif   | 172            | 55–65             |
| 82  | CSWTA01      | AACATATTCAATAAACACATCT               | AGGTTTTCCCTAATGATGAA             | (TA)7                  | 254            | 45–60             |
| 83  | CSWTA03      | ATGTGATTACATGCTGGAGCTATA            | AAAACCAAGAATATTGTTGAATAG          | (TA)10(TA)20           | 413            | 55–68             |
| 84  | CSWTA04      | TAAACATAATGTGATTATACTAGC            | GATGTTTGGTTGTTGTTGAGATATC        | (A)11(TA)10           | 314            | 51–54             |
| 85  | CSWTA05      | GCATGAGGCTCGAGCTGTTGAGTG            | GTCGAGCTTCTCTTGTTGAGGTAAT         | (TA)12                  | 278            | 57–60             |
| 86  | CSWTA06      | GAATTTAAAAATTTGATGGCTAT              | GAATTTAAAAATTTGATGGCTAT          | (TA)7(TA)16           | 292            | 55–65             |
| 87  | CSWTA07      | TGCGATTTGAGACACCCTTTATGAT            | TGCGATTTGAGACACCCTTTATGAT         | (TA)4(TA)7            | 355            | 53–65             |
| 88  | CSWTA08A     | AAAGTGGGCGACTCATGGGTAAA              | AAAGTGGGCGACTCATGGGTAAA          | (T)8(A)13(TA)6        | 347            | 45–60             |
Table 1 (continued). DNA sequences, optimal annealing temperature (°C), and expected product size (EPS) (bp) for 110 SSR and two SCAR markers characterized in cucumber (*Cucumis sativus* L.).

| No. | Marker | Sense primer 5’ to 3’ (top) | Antisense primer 5’ to 3’ (bottom) | SSR motif | EPS (bp) (top) | Temp °C (bottom) |
|-----|--------|---------------------------|-----------------------------------|-----------|----------------|-----------------|
| 89  | CSWTA08B | TTGCATTAATGCTATACCTTACC    | GAAATTAATTTTGGCATTG              | (T)7(TA)7 IN-DEL | 630            | 54–56           |
| 90  | CSWTA08C | TAATATTGCACTAGTTAGTATGCT   | GAAATTAATTTTGGCATTG              | (T)7(TA)7 | 556            | 53–65           |
| 91  | CSWTA09  | TCAATTTTCACTTCATCCCTATTT   | TCTAATTGATATTTTGGCATTG           | (TA)4(TG)3(TA)6 | 476            | 55–65           |
| 92  | CSWTA10  | TATAGGAGGACCAGCTTACCCAGC   | ACTCACTGCTCACCCTTATCCAGA         | (TA)4(TTAA)3(T)9 | 435            | 55–65           |
| 93  | CSWTA11B | GTAGGACCAATCAAGAGTGAGAGGT  | SACATATAGGAATAACTAAAAGTTG        | (TA)10(CA)7(TA)5 | 390            | 53–60           |
| 94  | CSWTA11C | GTCATAAGCTAAAGATATGCTTTCC  | ACTCTCACTCTTGTCACTTTCCTCCTC     | (TA)8     | 564            | 45–56           |
| 95  | CSWTA13  | AGATGGGAGGATTAGTATGATGCT   | GATTAAAAATTGATATGATGATGATG       | (TA)8 (GGTT)4 | 303            | 53–65           |
| 96  | CSWTA14  | GAATTTCAAACAGATTTCTGATTTG  | GAATTTCAACATTACATAAAATCAAT       | (TA)6     | 301            | 53–65           |
| 97  | CSWTA15  | GAAATTCAAGCATTTATTTTATTTTA | GAAATTCAAGCATTTATTTTATTTTA       | (TA)8     | 239            | 47–65           |
| 98  | CSWTA16  | TATGCAACCTTCTTTTGACGATTT   | CATTGCCACCTTCAAGCAGACTTCAATTC    | (TA)6,(TA)3 | 332            | 45–60           |
| 99  | CSWTA17  | CTGAATGGAGACTCTTTTCTAATC   | CATGATTCAGGAATGCTAAATGTAATAC    | (TA)6(TT)3(TA)6(T)11 | 198            | 45–60           |
| 100 | CSWTA1A01| CAATTTCCTAATCTGATAGGAGAG  | ACTGAGGTCTCACTATTTGTTGAGG        | (TAAA)4   | 306            | 57–65           |
| 101 | CSWTG01  | ATTTGCACTCTGAGTGATGATG     | GAGCAAGAGGAGGACTTACC             | (TG)5     | 178            | 45–65           |
| 102 | CSWTG02  | CTGAATTGCAACGTTTTTTTG     | AAGAAAAGGCTCAGGAGATG             | (TG)3-(TG)4 | 240            | 58–65           |
| 103 | CSWTG03  | GAATTTCAAAGAACATTTCCTATT   | GAATTTCAAAGAACATTTCCTATT         | (T)13(TG)7(T)7 | 273            | 52–65           |
| 104 | CSWTG04  | GAATTTCAAGAACATTTCCTATT   | GAATTTCAAGAACATTTCCTATT         | (TG)9     | 201            | 52–57           |
| 105 | CSWTG06  | ATGAAATGATGCTTACAGTACAT   | GAATTTCAAGAACATTTCCTATT         | (CA)5(GAAA)4 | 344            | 45–65           |
| 106 | CSWTG07  | GAAATTTGAAGAATCCTATCACAT   | GAAATTTGAAGAATCCTATCACAT         | (CA)5(GCA)4(N)(CA)3 | 249            | 68–72           |
| 107 | CSWTG08  | ATTGCACATCTGGTGCTCCTGCG   | ATTCACACTTCAGTCTCTGCTCTGCTCTGCT | (TG)5N3(TG)4(TGCG)5 | 246            | 68–72           |
| 108 | CSWTG09  | GAATTTTCACTATATATATTTTAT   | GAATTTTCACTATATATATTTTAT         | (TA)5(CA)11 | 262            | 45–65           |
| 109 | CSWTG10  | TGTGGACAGATGTTGAGTTGAGTTG | TGTGGACAGATGTTGAGTTGAGTTG        | (TG)9     | 252            | 45–60           |
| 110 | CSWTG11  | CTGGGAGGAGCTGTCACTACAG    | CTTTGCGGAGGAGCTGTCACTACAG        | (TG)6(TG)4(TGCC)6 | 216            | 68–72           |
| 111 | N6-1RTRANS | TCTATGATTTTCAAAATTGGGAGAAGG | TCTATGATTTTCAAAATTGGGAGAAGG     | SNP       | 50–56          | Retrotransposon  |
| 112 | L18-2 H19A | CCATCTAGTCAATGAAATAGGAATGA | CCATCTAGTCAATGAAATAGGAATGA      | SNP       | 50–56          | 5’ flanking region |

*Primer redesign based on empirical results from previous primer sets.*
30% of the total nuclear DNA in cucumber. Highly repetitive satellite DNA families represent about 20% to 25% of the genome. Digestion of the Lambda ZAP arm libraries in SOC media for >60 min allowed for one to two cell divisions in DH10B E. coli cells. This interval may have resulted in the duplication to quadruplication of a single capture product. This hypothesis is supported by the fact that in a second capture experiment using the same library and oligonucleotide where the incubation time was shortened to 25 min, redundancy was reduced from 65% to 10%. Last, single-stranded contaminants which were not eliminated during washing steps after the streptavidin capture were likely retained in the final solution used to electroporate competent cells. Given the representational bias of Type I and Type II satellite DNA in genomic libraries, this satellite DNA was likely also represented in the captured clones. Moreover, the redundancy of clone sequences was observed in subsequent captures (data not presented).

The application of the GeneTrapper system made it possible to isolate and characterize several cucumber microsatellite loci in a relatively short time without the use of radiolabeled products. This GeneTrapper protocol first described by Fazio and Staub (2000) in a preliminary report was also used to isolate sequences containing tandem repeats in a catfish (Ictalurus punctatus) brain cDNA library (Nonneman et al., 2001) where a redundancy of only 30% was observed. This capture reportedly yielded 64% (299/467) nonredundant clones, all containing microsatellites. Their experiments yielded a higher number of microsatellite containing clones (299) than what was obtained in the capture of cucumber microsatellites. This difference is likely due to efforts to increase genome representation in the 200 to 1200 bp range, which consequently decreases efficiency in cucumber. Future attempts to use this methodology should take into consideration the presence of repetitive sequences when constructing a genomic library.

The capture and identification protocol described herein was used to develop 110 microsatellite markers (Table 1). Of these markers, 38 (52%) contain dinucleotide repeats (CT, CA, TA), 21 (19%) possess trinucleotide repeats (CTT, ATT, ACC, GCA), 3 (2.7%) contain tetranucleotide repeats (TGGC, TTAA, TAAA), 4 (3.6%) enclose pentanucleotide repeat (ATTTT, GTTTT, GGTTT, AGCCC), 3 (2.7%) contain hexanucleotide repeats (CCCCAA, TAAAAA, GCTGGC), and 21 possess composite repeats. All primers amplified the expected product and were subjected to ATG-PCR to identify optimized conditions for PCR.

Optimization of primer-pair-specific PCR conditions. The potential of microsatellites and other PCR based markers in high throughput applications has been widely discussed (Donini et al., 1998; Gupta and Varshney, 2000; Powell et al., 1996). Multiplexing (i.e., the addition of multiple markers in a single PCR amplification) has been successfully used for parentage testing in goats (Capra hircus L. Bovidae) using two multiplex systems, each containing 11 microsatellite loci (Luikart et al., 1999). Multiplexing has also been used in several plant species including wheat (Triticum sp.) (Donini et al., 1998; Gupta et al., 1999), apple (Malus x domestica Borkh.) (Hokanson et al., 1998), and grape (Vitis sp.) (Lamboy and Alpha, 1998). However technical problems can arise during multiplexing. Donini et al. (1998) reported that multiplex PCR was very dependent on the choice of primer combinations and seldom produced amplifications as consistently as when primer pairs were used individually, and that background (nonspecific) amplification was common to many primer pairs, thus hindering the use of multiplex PCR. Furthermore, preferential amplification of smaller alleles over larger alleles as well as the variable plus A modification (nontemplated adenylation of the 3' end of the amplified sequence by Taq polymerase) resulted in misidentification of certain rainbow trout genotypes (Oncorhynchus mykiss, Salmonidae, Walbaum 1792) (Fishback et al., 1999). During PCR each primer pair may yield several products depending on reaction conditions (i.e., PCR mix, thermal cycler, and cycling conditions) (Staub et al., 1996a), and the nature of the DNA region that is being amplified (i.e., repeated

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Fig. 2. Annealing temperature gradient (ATG) profile of CSWTAAA01 SSR marker using DNA of cucumber lines H-19 and G421 alternating in paired comparison. Lane 1 contains a 100-bp ladder and annealing temperature increases from 45 °C (far left) to 65 °C far right with a step of 1.8 °C every two wells.

or unique) (Paglia and Morgante, 1998). Primer-pair characteristics may be such that they hinder PCR (i.e., reaction kinetics) resulting in inadequate resolution of predicted multiplexed primer products.

This and other problems associated with multiplexing reactions (i.e., reaction optimization) could result in inconsistent and inefficient implementation of this PCR application where equipment and reagents are not standardized. Implementation difficulties may be minimized by performing ATG-PCR profiling for each primer using standard DNA preparations (Fig. 2). The application of ATG-PCR as a preparatory step to PCR, assists in reaction optimization by identifying low risk temperature dependent annealing regions in which only a single target amplicon is present after PCR. Thus, the ATG-PCR profiling of potential PCR-based markers such as those developed herein (Table 1) is necessary for their universal acceptance and utility.

Observation of 214 SSR primer-pair annealing temperature optimizations (45 to 65 °C) using cucumber DNA (lines H-19 and G421) revealed four consistent visual band grouping types: 1) amplification of a single ATG-PCR product (=25% of the primer pairs) (Fig. 3, panel A); 2) amplification of multiple products at lower temperatures and the presence of a single PCR product at higher temperatures (=40% of the primer pairs) (Fig. 3, panel B); 3) amplification of multiple PCR regardless of annealing temperature (=25% of the primer pairs) (Fig. 3, panel C), and; 4) amplification of single or multiple PCR products only at relatively high temperatures (≥60 °C; =10% of the primer pairs) (Fig. 3, panel D).

These ATG-PCR profiling observations can be used to strategically multiplex SSR or SCAR primers, identify new polymorphisms, and avoid mislabeling of amplicons. For instance, ATG-PCR profile outcome information can be used to group primer pairs to set optimized temperature conditions for singlet or multiplexed PCR. For multiplexing, markers of differing molecular weight can be grouped by optimal annealing temperature to increase the success of the PCR. Moreover, ATG-PCR profiling provides evidence of primer PCR temperature complexity that can be used by laboratories wishing to use particular markers. New polymorphisms were observed that were not expected based on primer ATG-PCR profiles (data not presented). These polymorphisms may be due to duplicated DNA regions in the cucumber genome.

Development of PCR markers based on single nucleotide polymorphisms (SNP) and characterized sequences (SCAR)

The conversion of randomly amplified targeted marker bands (RAPDs) to sequence specific PCR markers results in more stable markers (Staub et al., 1996c). Such conversion is often accomplished by cloning and sequencing polymorphic target bands. Resulting primers are designed specifically for a particular sequence (e.g., SCARS, STS) (Michelmore et al., 1991; Paran and Michelmore, 1993). This procedure was used in cucumber (Horejsi et al., 1999) in an attempt to convert 75 RAPD into SCAR
markers. Conversion resulted in 48 (64%) SCARs, in which 11 (15%) retained their polymorphic nature.

Molecular markers tightly linked to economically important traits such as disease resistance, yield or quality are of particular importance to cucumber improvement programs. Given the low level of polymorphism in cucumber (3% to 8%), the problematic nature of RAPD markers (reproducibility across laboratories; Staub et al., 1996a), and the low percentage of RAPD to SCAR conversion (Horejsi et al., 1999), the identification and development of methodologically stable markers is of consequence. Moreover, concomitant assessment of ATG-PCR profiles of putatively useful PCR markers increases their potential implementation.

**Conversion of SCAR L18to an SNP marker.** The SCAR, L18, is of particular importance since it is associated (LOD 10.4) with quantitative trait loci (QTL) for multiple lateral branching (MLB), an important yield component trait in cucumber (Serquen et al., 1997). In contrast to its source RAPD, however, this marker was monomorphic with respect to the mapping parents H-19 and G421. Its RAPD counterpart is linked in repulsion phase to the MLB QTL limiting its usefulness in marker-assisted backcross selection. Sequencing of monomorphic L18 SCAR products in this study revealed several single nucleotide polymorphisms (SNPs) within the amplicon (data not presented). Consequently, both sense and antisense primers were designed so that the 3’ end of the oligonucleotides matched only the SNP allele evident in H-19. Since alternative SNPs were identified, two unique markers (L18-2-H19A and L18-2-H19B) were created. Marker L18-2-H19A was designed on a five-base polymorphism (i.e., two-base identity, and two-base Indel in the forward primer and one-base identity in the reverse primer). Marker L18-2-H19B SNP was based on one-base polymorphisms in both forward and reverse primers. These primer constructions were examined by the ATG-PCR protocol (Fig. 4) to determine their reliability in differentiating H-19 and G421 DNA, and to optimize reaction conditions. Results indicate that the SNP marker construction based on multiple polymorphisms (L18-2-H19A) was more reliable in a broad PCR temperature range than the marker based on a single base pair change (L18-2-H19B) (data not presented). Thus, L-18-H19A was successfully used in marker-assisted selection and quantitative trait analysis experiments with cucumber (Fazio, 2001). Sequencing of RAPD bands L18-3 and AT1-2 resulted in the construction of two codominant SCAR markers, L18-3-SCAR and AT1-2-SCAR (Table 1).

**Conversion of RAPD N6to SCAR N6.** The RAPD to SCAR conversion of a codominant RAPD product, N6, resulted in the characterization of a SCAR marker designated SCAR N6 (Horejsi et al., 1999). Although this SCAR yielded different size bands in both H-19 (584 bp) and G421 (1046 bp), analysis of a F2 mapping population (Fazio, 2001) revealed that this marker behaved in a dominant fashion exhibiting preferential amplification of the 584 bp band in heterozygous individuals. An ATG-PCR profile of the original N6 SCAR primers revealed novel polymorphisms between 45 °C to 48 °C. These were subsequently mapped (Fazio, 2001), and found to be distant (50 cM) from the N6 SCAR.

Given the size difference between the two N6 alleles (462 bp), it is possible that competition in the PCR reaction caused the higher molecular weight band to be amplified less efficiently than its smaller weight counterpart in heterozygous DNA samples. Sequence analysis (data not presented) of both alleles revealed an insertion of a transposable element belonging to the SINE-like family of retrotransposons (Kumar and Bennetzen, 1999; Kunze et al., 1997). These elements share features with SINE and Alu sequences in mammals, and are characterized by flanking 14 to 15 bp target-site duplications (TSDs) and a GC rich region in the 5’ half of the insert. The element from cucumber contains a 90-bp GC rich region in one of the termini as well as 17-bp long TSDs (data not presented). The GC rich region might have contributed to the failed amplification of the 1046-bp band of N6 SCAR marker in heterozygous individuals by raising the Tm requirement of the amplicon during PCR.

A strategy was used to convert this pseudodominant N6 SCAR marker into a codominant marker. Three primers were constructed resulting in a forward and reverse primer which amplified the putative nonretrotransposon allele and an additional reverse primer 30 bp within the putative retrotransposon insertion further away from the GC rich region. Genetic analysis of these primers using an F2 population (H-19 × G421) demonstrated that the new SCAR marker (N6-SCAR) segregated in a codominant fashion (Fazio, 2001). Sequences from plant retrotransposons elements have been used in Pisum species and conifers to construct PCR-based marker systems (Flavell et al., 1998; Pearce et al., 1999) for use in high throughput analysis. The development of markers based on retrotransposon sequences such as N6 will provide additional markers for genetic analysis in cucumber.

The application of molecular markers may increase the efficiency of plant breeding (Gupta and Varshney, 2000; Lande, 1990; Lee, 1995; Staub et al., 1996c; Young, 1999). Marker systems developed for the genetic analysis of cucumber have provided valuable information about genetic diversity (Dijkhuizen et al., 1996; Horejsi and Staub, 1999; Staub et al., 2002), have enhanced genetic map construction (Bradeen et al., 2001), and have been used in marker-assisted selection (Fazio, 2001). The level of polymorphism in a preliminary assessment of SSR variation between C. sativus var. sativus and var. hardwickii and among elite germplasm (European and U.S.) is ≈35% and =15%, respectively (unpublished data). Thus, the addition of 110 SSR, 4 SCAR and 2 SNP markers to the array of previously identified markers will enhance the use of genetic markers in breeding, diversity analysis, variety identification, and in the protection of cucumber germplasm. Moreover, these markers will likely provide a means for greater integration of genetic information during mapping experiments in cucumber (e.g., map merging) and in elucidating syntenic relationships between cucumber and melon (Cucumis melo L.).

**Literature Cited**

Anderson, B. and G. McDonald. 1993. Construction of DNA libraries of A-T rich organisms using EcoRI star activity. Anal. Biochem. 211:325–327.

Bradeen, J.M., J.E. Staub, C. Wye, R. Antonise, and J. Peleman. 2001. Towards an expanded and integrated linkage map of cucumber (Cucumis sativus L.). Genome 44:111–119.

Breslauer, K.J., R.F. Frank, H. Blocker, and L.A. Marky. 1986. Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA 83:3746–3750.

Bryan, G.J., A.J. Collins, P. Stephenson, A. Orr, J.B. Smith, and M.D. Gale. 1997. Isovalue and characterization of microsatellites from hexaploid bread wheat. Theor. Appl. Genet. 94:557–563.

Buteler, M.I., R.L. Jarret and D.R. LaBonde. 1999. Sequence characterization of microsatellites in diploid and polyplid Ipomea. Theor. Appl. Genet. 99:123–132.
retrotransposon LTR sequences for molecular marker studies. Plant J. 19:711–717.

Powell, W., M. Morgante, C. Andre, M. Hanafey J.-Vogel, S. Tingey, and A. Rafalski. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breeding 2:225–238.

Quarrie, S.A. 1996. New molecular tools to improve the efficiency of breeding for increased drought resistance. Plant Growth Regulat. 20:167–178.

Robinson, C.R. and S.G. Sligar. 1995. Heterogeneity in molecular recognition by restriction endonucleases: Osmotic and hydrostatic pressure effects on BamHI, PvuII, and EcoRV specificity. Proc. Natl. Acad. Sci. USA 92:3444–3448.

Roder, M.S., V. Korzun, K. Wendehake, J. Plaschke, M.H. Tixier, P. Leroy, and M.W. Ganal. 1998. A microsatellite map of wheat. Genetics 149:2007–2023.

Romagosa, I., F. Han, S.E. Ullrich, P.M. Hayes, and D.M. Wesenberg. 1999. Verification of yield QTL through realized molecular marker-assisted selection responses in a barley cross. Mol. Breeding 5:143–152.

Rychlik, W. and R.E. Rhoads. 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. Nucleic Acids Res. 17:8543–8552.

Rychlik, W., W.J. Spencer, and R.E. Rhoads. 1990. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res. 18:6409–6412.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.-B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Serquen, F.C., J. Bacher, and J.E. Staub. 1997. Mapping and QTL analysis of horticultural traits in a narrow cross in cucumber (Cucumis sativus L.) using random-amplified polymorphic DNA markers. Mol Breeding 3:257–268.

Shen, L., B. Courtois, K.L. McNally, S. Robin, and Z. Li. 2001. Evaluation of near isogenic lines of rice introgressed with QTLs for root depth through marker-aided selection. Theor. Appl. Genet. 103:75–83.

Staub, J.E. and V. Meglic. 1993. Molecular genetic markers and their legal relevance for cultivar discrimination: A case study in cucumber. HortTechnology 3:291–300.

Staub, J.E., J. Bacher, and K. Poetter. 1996a. Sources of potential errors in the application of random amplified polymorphic DNAs in cucumber. HortScience 31:262–266.

Staub, J.E., A. Gabert, and T.C. Wehner. 1996b. Plant variety protection: A consideration of genetic relationships. HortScience 31:1086–1091.

Staub, J.E., F.C. Serquen, and M. Gupta. 1996c. Genetic markers, map construction, and their application in plant breeding. HortScience 31:729–741.

Staub, J.E. 1999. Intellectual property rights, genetic markers, and hybrid seed production. J New Seeds. 1:39–64.

Staub, J.E., F. Dane, K. Reitsma, G. Fazio, and A. Lopez-Sese. 2002. The formation of test arrays and a core collection in cucumber using phenotypic and molecular marker data. J. Amer. Soc. HORT. Sci. 127(4):558–567.

Tanksley, S.D. and J.C. Nelson. 1996. Advanced backcross QTL analysis—A method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor. Appl. Genet. 92:191–203.

University of Arkansas–Fayetteville. 1993. H-19 cucumber plant variety Protection certificate, PVP no. 8900073. USDA–Agr. Mktg. Serv., Wash., D.C.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Mol Breeding 5:505–510.

Yousef, G.G. and J.A. Juvik. 2001. Comparison of phenotypic and marker assisted-selection for quantitative traits in sweet corn. Crop Sci. 41:645–655.

Yu, K.F., S.J. Park, and V. Poysa. 1999. Abundance and variation of microsatellite DNA sequences in beans (Phaseolus and Vigna). Genome 42:27–34.