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Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce

I. Paran* and R. W. Michelmore

Department of Vegetable Crops, University of California, Davis, CA 95616, USA

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Summary. Sequence characterized amplified regions (SCARs) were derived from eight random amplified polymorphic DNA (RAPD) markers linked to disease resistance genes in lettuce. SCARs are PCR-based markers that represent single, genetically defined loci that are identified by PCR amplification of genomic DNA with pairs of specific oligonucleotide primers; they may contain high-copy, dispersed genomic sequences within the amplified region. Amplified RAPD products were cloned and sequenced. The sequence was used to design 24-mer oligonucleotide primers for each end. All pairs of SCAR primers resulted in the amplification of single major bands the same size as the RAPD fragment cloned. Polymorphism was either retained as the presence or absence of amplification of the band or appeared as length polymorphisms that converted dominant RAPD loci into codominant SCAR markers. This study provided information on the molecular basis of RAPD markers. The amplified fragment contained no obvious repeated sequences beyond the primer sequence. Five out of eight pairs of SCAR primers amplified an alternate allele from both parents of the mapping population; therefore, the original RAPD polymorphism was likely due to mismatch at the primer sites.

Key words: Molecular marker – Disease resistance – Lettuce – Downy mildew

* *Present address:* Department of Field Crops, The Hebrew University of Jerusalem, Rehovot, P.O. Box 12, 76100, Israel
Correspondence to: R. W. Michelmore

Introduction

Our long-term goal is to characterize the molecular mechanisms that determine specificity in plant-pathogen interactions. As the products of plant resistance genes have not been identified, nor their expression determined, we are undertaking a map-based approach to clone genes for disease resistance in lettuce (Michelmore et al. 1992). A prerequisite for such an approach is a genetic map saturated with molecular markers in the regions containing resistance genes. Restriction fragment length polymorphisms (RFLPs) have been used to construct genetic maps in several crop species (O'Brien 1990). RFLPs can be used to initiate chromosome walks to clone genes in complex genomes (Gessler et al. 1990; Rommens et al. 1989). However, RFLP analysis is laborious and time-consuming and, therefore, methods are required for rapidly obtaining markers linked to resistance genes for genetic analysis and for physically characterizing the region.

Recently, PCR-based genetic markers have become available. These markers have been identified by either specific primers determined from known DNA sequences or by arbitrary primers. The former include allele-specific primers (Wu et al. 1989) and primers flanking minisatellites and microsatellites (Horn et al. 1989; Weber 1991). The widespread use of these types of markers is limited, however, because they rely on predetermined variation or on the genomic distribution and organization of tandem repeats. Many minisatellite alleles are too large to be amplified, and the microsatellites identified so far are only sparsely distributed in plant genomes (Weber 1991). Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) are identified by using arbitrary primers and allow the quick construction of genetic maps for any plant species or the saturation of specific genomic regions with molecular markers (Martin et al. 1991; Paran et al. 1991; Michelmore et al. 1991). Each amplification often detects five to ten RAPD loci. RAPD analysis is simple, fast, does not involve the use of radioactive isotopes,

and can be scaled up to analyze large numbers of samples. In order to utilize markers identified by RAPD analysis for map-based cloning, a single locus must be identified unequivocally. RAPD amplified products often contain repetitive DNA sequences and therefore can not be used an hybridization probes. In addition, the RAPD technique is sensitive to changes in the reaction conditions. There is, therefore, a gap between the ability to obtain linked markers to a gene of interest in a short time and the use of these markers for map-based cloning approaches and for routine screening procedures.

To address these problems we developed sequence-characterized amplified regions (SCARs) as PCR-based genetic markers. A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primers. We derived SCARs by cloning and sequencing the two ends of the amplified products of RAPD markers. The sequence was used to design pairs of 24-mer oligonucleotide primers that resulted in the reproducible amplification of single loci when high annealing temperatures were used. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into codominant markers. SCARs are similar to the sequence-tagged site (STSs) that have been proposed by Olson et al. (1989) as DNA landmarks in the physical map of the human genome. A STS is characterized by a short single-copy DNA sequence that can be amplified by PCR from a genomic library or genomic DNA using specific oligonucleotide primers. STSs allow communication between laboratories that is independent of the specific method employed in the physical mapping. In addition, the publication of STS sequences eliminates the need to store and distribute large numbers of clones. Physical mapping using STSs may be done by PCR (Green and Olson 1990) or by hybridization as STSs, at least by their original definition, do not contain repetitive DNA. While SCARs share the advantages of STSs, they are distinct from the latter in two aspects. SCARs are primarily defined genetically; therefore, they can be used not only as physical landmarks in the genome but also as genetic markers. In addition, SCARs can contain repetitive DNA sequences within the amplified fragment as they are analyzed by PCR only; their uniqueness is determined by the sequence and spacing of the primer sequences, rather than by hybridization.

In this paper we describe the development of SCARs linked to genes for resistance to downy mildew (*Dm*) in lettuce. RAPD markers linked to *Dm* genes have been previously identified using near-isogenic lines or bulked segregant analysis (Paran et al. 1991; Michelmore et al. 1991). Nearly all of the amplified fragments of the RAPD markers linked to *Dm* genes contained repetitive DNA sequences, which excluded their use as hybridization probes. Therefore, SCARs were derived from these RAPD markers to allow us to analyse the region physically. The SCARs will also be useful as genetic markers for high resolution mapping of the *Dm* regions and for plant breeding applications such as marker-aided selection and cultivar identification. In addition, this study provided information on the basis of RAPD polymorphisms in lettuce and on the nature of the RAPD primer sites.

Materials and methods

Plant materials and linkage analysis

The near-isogenic lines used to identify RAPD markers linked to the *Dm* genes were as described by Paran et al. (1991). Two segregating populations were used to map the *Dm* genes and the linked markers. (1) A F_2 population of 66 plants from a cross between a butterhead cultivar, 'Kordaat', and a crisphead cultivar, 'Calmar' (Landry et al. 1987). This is the main mapping population being used to construct the genetic map of *Lactuca sativa* L. (2) A F_2 population of an inter-specific cross between *L. sativa* cv 'Vanguard 75' and *L. saligna* L. UC82US1. Linkage analysis was as described by Paran et al. (1991). Data were analyzed using the program. MAPMAKER (Lander et al. 1987). Linkage was considered significant if the LOD score was equal to or greater than 3.

Cloning and sequencing RAPD products

RAPD reactions were performed and analyzed as described by Paran et al. (1991). The amplified products of the linked RAPDs were excised from agarose gels, and the DNA was purified by the 'Geneclean' kit (Bio 101 Inc). The purified DNA was reamplified using the same primer that identified the RAPD polymorphism. After reamplification, the $MgCl_2$ concentration of the PCR mixture was adjusted to 5 m*M*, and 5 units of Klenow fragment DNA polymerase were added. The reaction was incubated at room temperature for 20 min and was stopped by heating the sample to 75 °C for 10 min. The amplification products were resolved by gel electrophoresis, excised from the gel, purified by the 'Gene Clean' kit, and blunt-end ligated into the pBluescript IIsk vector (Stratagene) that had been linearized with *Eco*RV. The identity of the cloned RAPD products was verified by hybridization of the cloned fragments to Southern blots of F_2 individuals that segregated for the progenitor RAPD marker. Double-strand sequencing (Sequenase kit USB) was done by the dideoxy-chain termination method using the M13 universal and T3 primers.

SCAR design and analysis

For each cloned RAPD amplification product, two oligonucleotides were designed to be used as SCAR primers. Each primer contained the original 10 bases of the RAPD primer plus the next 14 internal bases from the end. Primers were synthesized by and are available from Operon Technologies (Alameda, Calif.). Amplification of genomic DNA (1 ng/μl) with SCAR primers was done in a standard PCR reaction (Maniatis et al. 11989) and consisted of 30 cycles of 1 min at 94 °C, 1 min at 60 °C the exception being 67 °C for $SCM05_{410}$) and 2 min at 72 °C. Amplification products were resolved electrophoretically in a 2% agarose gel.

Results

Identification of RAPD markers linked to the Dm1 region

Five RAPD markers linked to the *Dm1* region were identified that had not been reported previously (Paran et al. 1991): $OPM05_{410}$, $OPV12_{330}$, $OPX11_{860}$, $OPW09_{860}$, and $OPQ05_{615}$ (Fig. 1). $OPM05_{410}$ was identified by screening for polymorphisms between pools of F_2 individuals differing for alleles of *Dm3* as described by Michelmore et al. (1991). All the other linked markers were identified by comparing near-isogenic lines that differed for *Dm1* and *Dm3* (Paran et al. 1991). The linkage map contains four RFLP and eight RAPD or SCAR markers within 41 cM in the region containing the major cluster of *Dm* genes (Fig. 2; Farrara et al. 1987). Two markers, $SCV12_{330}$ and $SCI11_{1200}$, were initially mapped as dominant RAPD markers, but later were converted to codominant SCAR markers (see below). All of the other RAPD markers were scored as dominant loci (presence or absence of an amplified band). Two markers, $OPX11_{860}$ and $OPJ11_{2000}$, absolutely cosegregated with *Dm3* in the progeny of 66 F_2 plants scored.

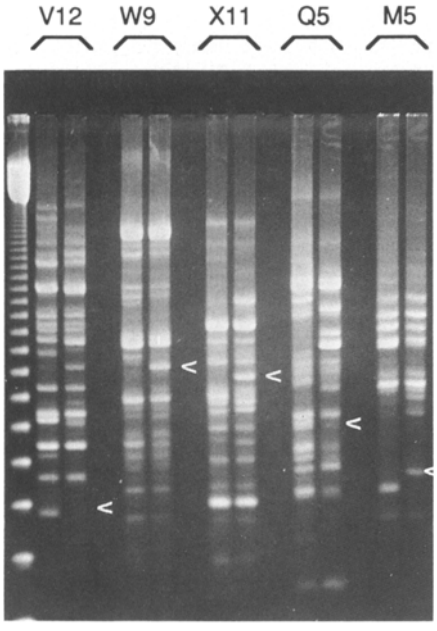

Fig. 1. Amplification products using RAPD primers to identify markers linked to *Dm1* and *Dm3*. The polymorphisms linked to the *Dm* genes are marked by *arrows*. *First lane* 123-bp molecular weight ladder. The first lane for each pair used 'Calmar' DNA as template and the second lane used 'Kordaat' DNA. Primers used were, from *left to right*: OPV12, OPW09, OPX11, OPQ05, and OPM05

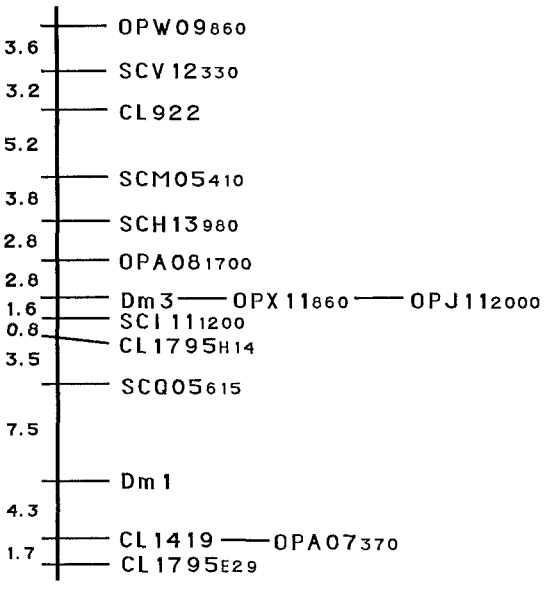

Fig. 2. A genetic map of the *Dm1* and *Dm3* region constructed from an intraspecific cross of *Lactuca sativa* cvs 'Calmar' × 'Kordaat'. A *CL* prefix indicates a locus detected by a cDNA clone, an *OP* prefix indicates a locus detected by a RAPD primer obtained from Operon Technologies; a *SC* prefix indicates a locus detected by a pair of SCAR primers. Genetic distances shown in cM are to the left of the vertical line

Cloning sequencing of RAPDs linked to Dm genes

The amplified products of nine RAPDs linked to *Dm* genes in three linkage groups, the *Dm1* region, *Dm4* region, and *Dm13* (Farrara et al. 1987), were cloned (Table 1). The identities of the cloned products were verified by hybridizations of the cloned fragments to Southern blots of individuals that segregated for that particular RAPD. The F_2 segregation of $OPM05_{410}$ and the hybridization pattern of the cloned fragment to a Southern blot of the same F_2 individuals is presented in Fig. 3. The hybridization pattern is identical to the RAPD segregation, indicating that the cloned fragment was derived from the $OPM05_{410}$ amplified product. This hybridization step was critical in verifying the identity of the cloned products. The RAPD reaction results in many fragments being amplified, and the region of the gel containing the polymorphic band may contain contaminating sequences of the same size at concentrations below the resolution of an ethidium bromide-stained gel. Positive clones cannot be identified by cross-hybridization to the amplified product because contaminating sequences can provide significant levels of hybridization signal if they contain repeated sequences. Of the clones initially selected 20% were not derived from the polymorphic RAPD fragment.

The cloned amplification products were sequenced for at least 200 bp from each end. The sequences at

Table 1. Sequence of 24-mer oligonucleotide primers for each SCAR locus derived from RAPD markers linked to genes for resistance to downy mildew in lettuce, and the type of polymorphism detected

Locus	Primer[a]	Sequence[b]	Polymorphism[c]	Linkage to *Dm* genes[d]
$SCA01_{860}$	$OPA01U_{860}$	CAGGCCCTTCGAATGTGAAAGACA	Dominant	*Dm4*
	$OPA01T_{860}$	CAGGCCCTTCCAGCTTTATCCTTT		
	$OPA01U_{450}$	CAGGCCCTTCAAGAATGTATCACG	None detected	*Dm13*
	$OPA01T_{450}$	CAGGCCCTTCCACACCGGATATAT		
$SCB12_{1100}$	$OPB12U_{1100}$	CCTTGACGCATACTATCGAGGTTT	Dominant	*Dm4*
	$OPB12T_{1100}$	CCTTGACGCACGAGATACTTCAGT		
$SCH13_{980}$	$OPH13U_{980}$	GACGCCACACACACACTTTAAGTA	Dominant	*Dm1*
	$OPH13T_{980}$	GACGCCACACCTATATCCTTTACC		
$SCI11_{1200}$	$OPI11U_{1200}$	ACATGCCGTGTATTACTCAGAGTT	Co-dominant with *Rsa*I	*Dm1*
	$OPI11T_{1200}$	ACATGCCGTGACAGTATGAGACCG		
$SCMO5_{410}$	$OPM05U_{410}$	GGGAACGTGTTAATTAGAGATGTA	Dominant at 67 °C	*Dm1*
	$OPM05T_{410}$	GGGAACGTGTGTGTGTATGGATCA		
$SCQ05_{615}$	$OPQ05U_{615}$	CCGCGTCTTGGGCTCGGCTCTTAG	Dominant	*Dm1*
	$OPQ05T_{615}$	CCGCGTCTTGCTGATCTTCATTGG		
$SCV12_{330}$	$OPV12U_{330}$	ACCCCCCACTACCATATCAATCTC	Co-dominant	*Dm1*
	$OPV12T_{330}$	ACCCCCCACTTGTCCTGCAACTTT		
$SCW09_{860}$	$OPW09U_{860}$	GTGACCGAGTAGTCTTAACCTAGT	Co-dominant	*Dm1*
	$OPW09T_{860}$	GTGACCGAGTGTAACAACGTAAAT		

[a] The third letter and the fourth and fifth numbers refers to the kit and primer number (Operon Technologies) used to identify the progenitor RAPD marker. The subscript number refers to the size in bp of the amplified RAPD product from either cv 'Calmar' or cv 'Kordaat'. These designations refer to the primers available commercially (Operon Technologies)

[b] The underlined sequences represent the sequence of the progenitor RAPD primers

[c] All polymorphisms identified at 60 °C reannealment except $OPM05_{410}$

[d] *Dm1* region includes *Dm1*, *Dm2*, *Dm3*, *Dm6*, *Dm14*, *Dm15*, *Dm16*; *Dm4* region includes *Dm4*, *Dm7* and *Dm11* (Farrara et al. 1987)

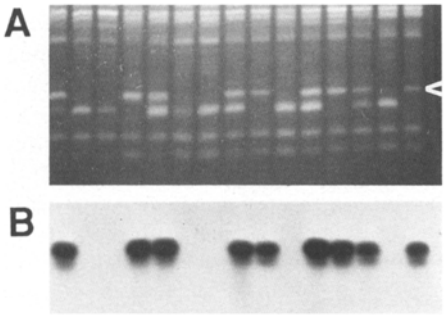

Fig. 3. Hybridization of the cloned RAPD product of $OPM05_{410}$ to a Southern blot of F_2 individuals segregating for OPM05. **A** Amplification products of F_2 individuals segregating for $OPM05_{410}$ (*arrowed*). **B** Hybridization of the amplification product of $OPM05_{410}$ to a Southern blot of the F_2 individuals shown in panel **A**

the two ends of the cloned RAPDs products did not reveal inverted repeats longer than the 10 bases consisting of the primer binding sites for each RAPD (Table 1). The terminal 10 bases exactly matched the primer sequences as the sequences were determined from the amplified products. These may not be the exact genomic sequences, any differences between primer and genomic sequences would have been lost after the initial rounds of amplification. The cloned RAPD fragments did not share similar sequences as shown by cross hybridizations of the cloned fragments and comparisons of their terminal sequences. One primer, OPA01, amplified three products, $OPA01_{450}$, $OPA01_{860}$, and $OPA01_{920}$, that were linked to *Dm13*, *Dm4*, and *Dm1* respectively; however, none of the three RAPDs shared similar sequences internal to the priming sites.

Amplification of genomic DNA using SCAR primers

For each cloned RAPD product, a pair of 24-mer SCAR primers was synthesized (Table 1). Genomic DNA from the parents of the mapping population. 'Calmar' and 'Kordaat', was used as the template for PCR amplifications with each pair of SCAR primers (Fig. 4). In each case (except $SCA01_{860}$), a single band of the same size as the progenitor RAPD fragment was amplified. In three cases, $SCH13_{980}$, $SCQ05_{615}$, and $SCB12_{1100}$, the RAPD polymorphisms were retained as the presence or absence of bands when the corresponding SCAR primers were used at an annealing temperature of 60 °C. These SCARs could be readily scored as dominant markers.

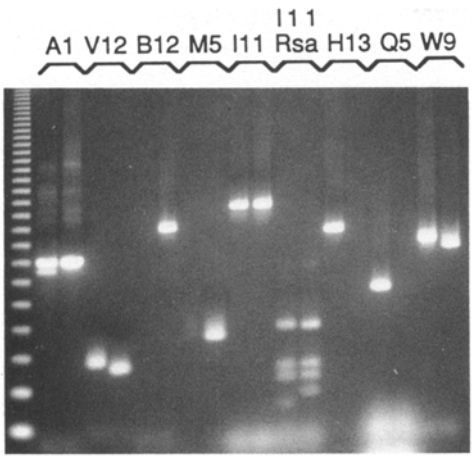

Fig. 4. Amplification of genomic DNA using SCAR primers. *First lane* 123-bp molecular weight ladder. SCAR primers, from *left to right*: $SCA01_{860}$, $SCV12_{330}$, $SCB12_{1100}$, $SCM05_{410}$, $SCI11_{1200}$, $SCI11_{1200}$ amplification products digested with *Rsa*I, $SCH13_{1100}$, $SCQ05_{615}$, $SCW09_{860}$. For all pairs except $SCW09_{860}$, the first land had 'Calmar' DNA as template, the second lane used 'Kordaat' DNA. For $SCW09_{860}$, the first lane used 'Vanguard 75' DNA, the second lane used *L. saligna*, UC82US1DNA

With six out of the nine pairs of primers, alleles were amplified from both parents, for three SCARs, $SCV12_{330}$, $SCI11_{1200}$, and $SCW09_{860}$, length polymorphisms resulted that made the SCARs codominant. Several strategies were used to identify polymorphisms that would allow confirmation of the genetic identity of the SCAR. Primers for $SCV12_{330}$ identified alleles of obviously different sizes, and therefore this locus could be readily scored as a codominant marker (Fig. 5). The remaining amplified SCAR fragments were digested with four different endonucleases (*Hae*III, *Mbo*I, *Rsa*I, *Alu*I) with four base-pair recognition sequences. This allowed us to search for restriction site differences and to divide the fragments into smaller

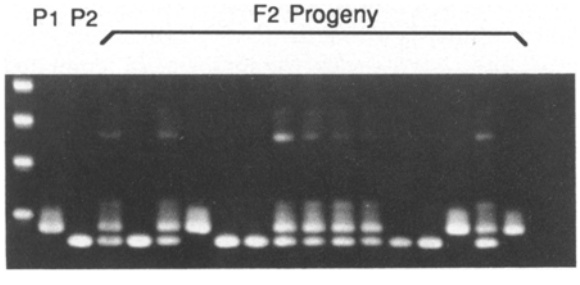

Fig. 5. Segregation of $SCV1_{2330}$ F_2 progeny from cvs 'Calmar' × 'Kordaat'. The *first lane* is a 123-bp molecular weight ladder, the *next two lanes* used parental DNA, 'Calmar' (P_1) and 'Kordaat' (P_2), as template. Heterozygous and both homozygous genotypes are clearly distinguishable in the progeny

pieces in order to facilitate the detection of small fragment length differences. In one case, $SCI11_{1200}$, digestion with *Rsa*I revealed a small fragment length polymorphism (Fig. 4) that had been too small to be resolved in a 2% agarose gel prior to digestion. For $SCW09_{860}$, polymorphism could not be detected between the amplification products from 'Calmar' and 'Kordaat'. Therefore, we searched for polymorphism between parents of different mapping populations. Fragment length polymorphism was detected between *L. sativa* cv 'Vanguard 75' and a wild accession of *L. saligna*, UC82US1 (Fig. 4). These two parents are being used to construct a map of this interspecific cross (R. V. Kesseli and R. W. Michelmore unpublished) and enabled $SCPW09_{860}$ to be mapped. We also tried elevating or reducing the annealing temperature during amplification. In no case did reducing the temperature reveal the alternate allele of a SCAR for which a band was amplified from only one parent at 60 °C. For $SCM05_{410}$, amplification occurred with both parents of 60 °C, but when the annealing temperature was increased to 67 °C, only the 'Kordaat' allele was amplified; therefore, this SCAR could be scored as a dominant marker. In the case of $SCA01_{860}$, in addition to a band of 860 bp, another band of 800 bp was amplified; this was the same size as the apparent alternate allele for the RAPD marker $OPA01_{860}$. Both fragments had previously been mapped and sequenced, and shown to differ by a 60-bp insertion. The molecular basis of the double-banded phenotype in 'Calmar' is unknown; however, the presence or absence of the 800-bp band detected with the SCAR primers for $SCA01_{860}$ cosegregated with the locus detected by the RAPD primers.

We failed to detect polymorphism using the 24-mer primers derived from the 'Kordaat' RAPD allele at $OPA01_{450}$. These primers amplified the same size product from several accessions of *L. sativa* and *L. saligna*. The allele amplified from 'Calmar' was sequenced in two separate clones; both clones provided the same sequence. We also sequenced the 'Kordaat' allele and identified two nucleotide differences between the 'Calmar' and 'Kordaat' alleles. These nucleotide changes resulted in restriction site polymorphisms, but these were for the commercially unavailable enzymes, *Bsa*HI and *Cvi*RI. We resolved the amplification products from 'Calmar' and 'Kordaat' using denaturating gradient gel electrophoresis; however, polymorphisms were not detected (data not shown). We could not therefore confirm that the 24-mer primers derived from $OPA01_{450}$ amplified DNA from a single locus. Consequently, the sequences identified from $OPA01_{450}$ are not considered to be a SCAR.

In summary, out of nine RAPD fragments cloned and sequenced, three resulted in SCARs that could be scored as codominant loci, five provided SCARs that

segregated as dominant markers, and one has yet to be converted to a SCAR due the lack of detectable polymorphism within the amplified fragment from diverse genotypes.

Determination of genomic copy number and origin of SCAR amplification products

To determine whether the SCAR amplification products contained repetitive DNA sequences, the amplified products were blotted and hybridized to a labelled lettuce genomic DNA. All of the SCAR amplification products, with the exception of that from $SCV12_{330}$, hybridized strongly (data not shown) and therefore contained repetitive DNA within the amplified regions. This was confirmed with Southern analysis of genomic DNA using the amplified products as probes; intense smears resulted. The amplification product from $SCV12_{330}$ showed no detectable hybridization to total labelled genomic DNA and when used as a probe on genomic Southerns detected only two bands of intensities similar to those of other single-copy sequences. Regardless of the genomic copy number within the amplified fragment, SCARs can be useful genetic markers as long as they originate from single loci, i.e., only one amplification site exists in the genome. To determine whether the SCARs represented single loci, their segregation was analyzed in F_2 progeny from the cross 'Calmar' × 'Kordaat' (except for $SCW09_{860}$, which was analyzed in the inter-specific cross between *L. sativa* and *L. saligna*). The F_2 segregation of $SCV12_{430}$ is shown in Fig. 5. Each SCAR cosegregated with the RAPD marker from which it was derived. Therefore, these SCARs represent single loci in an identical position to as that of the original RAPD markers.

Variability detected by SCARs

Genomic DNA from 48 lettuce cultivars and 15 accessions of three related *Lactuca* species were used as templates for PCR amplification with the SCAR primers. These lines were the same as those used for a previous taxonomic study employing RFLP markers (except that the accessions of *L. indica* and *L. perenis* used previously were omitted) and sampled the diversity within cultivated lettuce as well as closely related species (Kesseli et al. 1991). SCARs did not detect a high level of variation. For the dominant SCAR markers, $SCH13_{980}$, $SCQ05_{615}$, $SCM05_{410}$, and $SCB12_{1100}$, no additional alleles were detected as length variants in 2% agarose gels. With the codominant markers, amplification occurred in all of the accessions of *L. sativa* but only one additional length variant was detected for each of $SCA01_{860}$ and $SCV12_{330}$. For $SCA01_{860}$ and $SCV12_{330}$, no additional alleles were detected in the wild species. For $SCI11_{1200}$, an additional length variant allele was detected in *L. serriola*, while no amplification occurred in the more distantly related species, *L. saligna* and *L. virosa* (Fig. 6). No additional alleles were detected for $SCW09_{860}$.

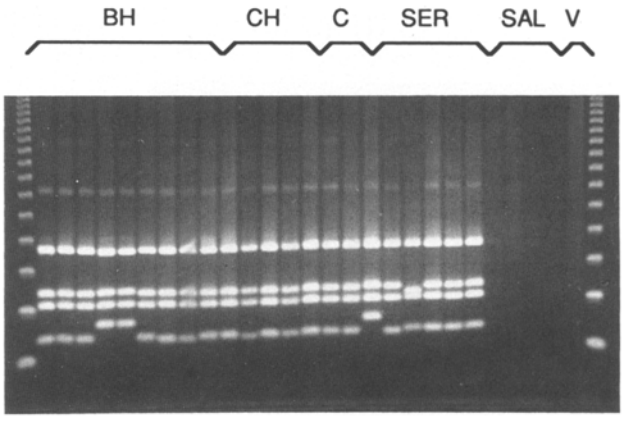

Fig. 6. Allelic variation detected at $SCI11_{1200}$ in *Lactuca sativa* and related species. The amplification products were digested with *RsaI* to reveal length polymorphisms. The *first* and *last* lanes are a 123-molecular weight ladder. *Lanes marked BH* had DNA from *L. sativa* butterhead cultivars as template; *lanes marked CH* used DNA from *L. sativa* crisphead cultivars; *lanes marked C* used DNA from *L. sativa* cos cultivars; *lanes marked SER* used *L. serriola* DNA; *Lanes marked SAL* used DNA from *L. saligna*; *lanes marked V* used DNA of *L. virosa*

Discussion

This study provides the first information on the molecular basis of the polymorphisms detected as RAPD markers. RAPD polymorphisms could be caused by differences in nucleotide sequence at the priming sites or by structural rearrangements within the amplified sequence. RAPD amplification can likely be initiated from genomic sites that do not perfectly match the primer sequence. Sequencing the products of RAPD analysis, however, does not provide information on mismatches within the RAPD primer sequence because, after a few cycles, the majority of templates will be identical in sequence to the primer. The amplification of alleles from both parents with six out of the nine pairs of 24-mer primers indicated that the original RAPD polymorphisms were caused by mismatches in one or a few nucleotides in the priming sites. These mismatches were tolerated by the longer primers, resulting in amplification from both lines. A precise assessment of the extent of the mismatches tolerated by the RAPD and SCAR primers will require cloning and sequencing of the genomic primer binding sites. For three SCARs, amplification occurred from only one of the parents used. The RAPD polymorphism

at these loci could have been caused by extensive sequence divergence or by rearrangements that either altered the orientation of the primers or resulted in the separation of the primers by too great a distance to allow amplification. No inverted repeats larger than the priming sites were detected within the amplified sequence. The existence of large inverted repeats would result in a non-random distribution of potential primer sites; therefore, the RAPD procedure would preferentially detect such sequences if they existed and could be amplified. These results suggest that contiguous inverted repeats are not frequent in the *Dm*-containing regions of the genome or such sequences are not readily amplified. Also, as the sequences obtained were only internal to the priming sites, the existence of inverted repeats external to the primer sites and separated by unrepeated sequences can not be excluded.

SCARs have several advantages over RAPD markers. The use of RAPDs allowed us to identify molecular markers linked to the *Dm* genes within a few months; however, their dominant nature, the amplification of multiple loci, and their sensitivity to reaction conditions restricted their further use. For RAPD polymorphisms that were caused by a mismatch in the priming sites, the use of the longer SCAR primers resulted in the amplification of products from both of the original parents. This allowed the conversion of three SCARs into codominant genetic markers. The mapping efficiency of RAPD markers in F_2 populations is decreased by their dominant nature, and statistically significant gene orders may be difficult to obtain for regions that contain multiple dominant markers. The conversion of dominant RAPDs to codominant SCARs increases the amount of information per F_2 individual. Therefore, the codominant SCARs are being used for a high resolution genetic mapping of the *Dm* region. As the annealing conditions are more stringent for SCARs than for RAPDs, only one locus was detected by the SCAR primers. Also, the use of longer oligonucleotide primers for SCARs allowed a robust and more reproducible assay than could be obtained with the short primers used for RAPD analysis. The will facilitate the use of molecular markers for other applications such as a marker-aided selection and fingerprinting. SCARs will also be useful in molecular taxonomy, since they may detect single loci that can be examined across species.

SCARs can be readily applied to commercial breeding programs as they do not require the use of radioactive isotopes. Although the codominant SCARs are the more useful for genetic studies, the dominant SCARs may be ultimately more useful in breeding applications if a quick plus/minus assay can be developed to detect the product. This would eliminate the need for electrophoresis to resolve the products as well as decreasing the cost and increasing the speed of the analysis. It would also remove the use of ethidium bromide, if less toxic colorimetric assays can be developed.

In order for a SCAR to be useful as a genetic marker or a physical landmark, it must be demonstrated that the primers identify only a single locus. Although the original RAPDs mapped to single loci, SCARs developed from them could be amplified from multiple loci. A RAPD primer may detect sequence differences among diverged repetitive DNA units, but the sequence mismatch may be tolerated by the longer SCAR primers. Therefore, each SCAR was defined genetically as a single locus by segregation analysis. This required that the amplification products of the 24-mer primers were polymorphic in a mapping population. Segregation of the eight SCARs demonstrated that they cosegregated with the RAPD markers from which they were derived. No polymorphisms could be detected in our mapping populations between the products of the extended primers derived from $OPA01_{450}$. Therefore, these products could not be demonstrated to be derived from a single locus; consequently, $OPA01_{450}$ is not currently considered to be a SCAR.

When the SCAR primers amplify the same sized fragment from both parents, various methods can be tried to identify polymorphism: (1) Increasing the annealing temperature may reveal different amounts of mismatch within a primer sequence. For $SCM05_{410}$, increasing the annealing temperature from 60 °C to 67 °C resulted in the loss of amplification of the 'Calmar' allele. (2) Polymorphisms can be searched for between more genetically diverged lines from which mapping populations have been derived. $SCW09_{860}$, was monomorphic in an intra-specific cross of the cultivated species but was polymorphic in an inter-specific cross. (3) Single base differences between two alleles might be identified by various methods such as denaturating gradient gel electrophoresis (Meyers et al. 1987) or ribonuclease cleavage (Meyers et al. 1988). (4) Sequencing of the alternate alleles might identify diverged regions for which it may be possible to design allele-specific primers. Alternatively, diagnostic restriction sites may be identified for the two alleles. Our inability to detect polymorphism with the 24-mer primers derived from $OP01_{450}$ may be due to the longer primers amplifying sequences from multiple sites in the genome.

The level of variation detected by the SCARs in the germplasm survey of *Lactuca* spp. was low. Only in one case, $SCI11_{1200}$, were new alleles detected in the wild species. This is in contrast to the level of variation detected by RFLPs in lettuce. Although the intra-specific variability within the cultivated species was similarly low, new alleles were often detected by RFLP probes in the wild species (Kesseli et al. 1991).

This difference probably reflects the ability of the two methods to detect divergent alleles. Several types of genetic changes that are detectable by RFLP analysis may prevent amplification by PCR. The majority of fragments amplified by the RAPD primers were smaller than 2 kbp, while RFLP probes detected length variation in fragments up to 10 times larger. Also, nucleotide changes in restriction sites usually result in the detection of both alleles; in contrast, variation in sequence at the SCAR primer sites may not result in differential amplification.

SCARs may identify polymorphisms that are less accessible by other techniques. The small fragments amplified allow the detection of chromosomal changes such as small insertions and deletions. Three insertions were detected by SCARs: $SCV12_{330}$, $SCW09_{860}$, and $SCI11_{1200}$. These insertions were not detected by the corresponding RAPD primers, since amplification of the RAPD band occurred from only one of the parents. Occasionally, alternate alleles can be detected by RAPD primers as was the case for the primer OPA01 that detected two alleles at 800 and 860 bp. If high resolution gels such as acrylamide are used, the size differences of few nucleotides should be resolved.

SCARs can be derived not only from RAPDs but also from cloned sequences such as RFLP probes. SCARs derived from RFLP probes will be identical to STSs but may not be useful as PCR-based genetic markers. The differences underlying RFLPs are often outside the regions hybridizing with the probe; these polymorphisms will not be detected by primers obtained from the two ends of the RFLP probe. Therefore, it may be difficult to identify polymorphisms within the regions amplified. Recently, random cDNA clones from a human brain library were sequenced and converted to STSs (Adams et al. 1991). However, cDNA sequences may be less useful than genomic clones as a source of SCARs because of introns. If the two primer sites flank an intron, the intervening fragment may be too big to be amplified; also if the sequence of an individual SCAR primer is interrupted by an intron, primer binding will be obstructed. Furthermore, coding regions may be more conserved than the random sequences identified from RAPDs.

In addition to their use as genetic markers, SCARs will be useful in physical mapping. SCARs will bridge the gap between the ability to obtain molecular markers linked to genes of interest in a short time and the use of these markers in a map-based cloning approach. In lettuce, the linked markers will be used for screening genomic libraries for physical mapping of the *Dm* regions and to obtain overlapping clones in the process of chromosome walking to clone the *Dm* genes. A partial YAC library of lettuce (M. G. Fortin and R. W. Michelmore, unpublished) will be pooled and screened (Green and Olson 1990) with the SCAR primers. The utility of a SCAR is increased if amplification occurs from all genotypes. If an allele is only amplified in some genotypes, only markers that are in *cis* to the target gene can be used for screening a library. Five out of the eight SCARs amplified sequences from both parents; consequently, they will be useful for screening all genotypes. The YAC library of lettuce was constructed from cv 'Kordaat' and, therefore, can not be screened by primers $SCH13_{980}$ and $SCQ05_{615}$ that do not amplify the alleles from 'Kordaat'.

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