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Conversion of AFLP fragments tightly linked to SCMV resistance genes *Scmv1* and *Scmv2* into simple PCR-based markers

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Abstract In a previous study, bulked segregant analysis with amplified fragment length polymorphisms (AFLPs) identified several markers closely linked to the sugarcane mosaic virus resistance genes *Scmv1* on chromosome 6 and *Scmv2* on chromosome 3. Six AFLP markers (E33M61-2, E33M52, E38M51, E82M57, E84M59 and E93M53) were located on chromosome 3 and two markers (E33M61-1 and E35M62-1) on chromosome 6. Our objective in the present study was to sequence the respective AFLP bands in order to convert these dominant markers into more simple and reliable polymerase chain reaction (PCR)-based sequence-tagged site markers. Six AFLP markers resulted either in complete identical sequences between the six inbreds investigated in this study or revealed single nucleotide polymorphisms within the inbred lines and were, therefore, not converted. One dominant AFLP marker (E35M62-1) was converted into an insertion/deletion (indel) marker and a second AFLP marker (E33M61-2) into a cleaved amplified polymorphic sequence marker. Mapping of both converted PCR-based markers confirmed their localization to the same chromosome region (E33M61-2 on chromosome 3; E35M62-1 on chromosome 6) as the original AFLP markers. Thus, these markers will be useful for marker-assisted selection and facilitate map-based cloning of SCMV resistance genes.

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Introduction

Sugarcane mosaic virus (SCMV) is one of the most important virus diseases of maize (*Zea mays* L.) and causes serious yield losses in susceptible cultivars (Fuchs and Grüntzig 1995). It is naturally transmitted by aphids in a non-persistent manner, which makes control of SCMV vectors rather inefficient. Therefore, cultivation of resistant varieties is the most promising approach for controlling of SCMV.

Kuntze et al. (1997) screened 122 early-maturing European inbred lines for resistance to SCMV and MDMV (maize dwarf mosaic virus) and identified three dent inbreds (D21, D32 and FAP1360A) displaying complete resistance under both field and greenhouse conditions. Two major genes, *Scmv*1 and *Scmv*2 (previously named *Scm1* and *Scm2*), conferring resistance to SCMV were mapped to chromosome arms 6S and 3L, respectively, in cross D145 × D32 by quantitative trait loci (QTL) analysis (Xia et al. 1999) and in cross F7 × FAP1360A by bulked segregant analysis (BSA) (Xu et al. 1999) and QTL analysis (Dussle et al. 2000). As resistance against SCMV is strongly affected by environmental conditions (Melchinger et al. 1998), molecular markers turned out to be a good tool to determine the resistance genotype.

Identification of molecular markers closely linked to the SCMV resistance genes is an essential step towards both marker-assisted selection (MAS) and map-based cloning of these genes. Xu et al. (1999) identified 23 tightly linked amplified fragment length polymorphism (AFLP) markers for both major resistance genes by BSA: 11 markers linked to *Scmv2* on chromosome 3 and 12 linked to *Scmv1* on chromosome 6, including one AFLP marker cosegregating with *Scmv1*.

Although the AFLP technique is powerful and reliable in identifying markers closely linked to genes of in-

terest, it has some disadvantages for use in MAS and map-based cloning. Limitations to the large-scale, locus-specific application of AFLPs include their dominant type of inheritance, the intensity of labour involved, and the high costs. Hence, conversion of AFLP markers into sequence-specific polymerase chain reaction (PCR) markers is required for screening large breeding populations at low costs.

Sequence-specific PCR markers have been successfully developed by conversion of different marker types such as RFLPs, RAPDs and SSRs, (Bradshaw et al. 1994; Cheung et al. 1997; Jung et al. 1999). The conversion of AFLP markers into PCR-based markers has been accomplished for several species such as carrot (Bradeen and Simon 1998), brassica (Negi et al. 2000), asparagus (Reamon-Büttner et al. 2000), soybean (Meksem et al. 2001), apple (Xu et al. 2001), barley and wheat (Shan et al. 1999). However, the conversion of AFLP markers seems to be more difficult than the conversion of other marker types due to the loss of their sequence specificity after amplification of the AFLP-derived internal primers (Shan et al. 1999). Hence, AFLP polymorphisms related to *Eco*RI or *Mse*I restriction site differences will not be reflected in primers from an internal sequence (Shan et al. 1999).

The objective of the study reported here was to sequence the respective AFLP bands linked to SCMV resistance genes in order to convert these dominant markers into either indel (insertion/deletion) or cleaved amplified polymorphic sequence (CAPS) markers. These AFLP markers were previously identified by BSA to be closely linked with *Scmv1* on chromosome 6 (two markers) or *Scmv2* on chromosome 3 (six markers). Our goal was to obtain codominant, simple PCR-based markers as a tool for marker-assisted selection as well as for map-based cloning of *Scmv1* and *Scmv2*.

Materials and methods

Plant materials

Inbred lines used in this analysis were the SCMV-resistant European inbred lines FAP1360A, D21 and D32 and the highly susceptible lines F7, D408 and D145. The mapping population consisted of (1) a subset 87 $F_{2:3}$ families derived from a cross between D32 and D145 previously used by Vuylsteke et al. (1999) to develop a high-density AFLP map (1,355 markers), and (2) 27 resistant BC_5 individuals from the cross (F7 × FAP1360A) × F7.

Isolation and cloning of tightly linked AFLP markers

AFLP markers flanking *Scmv1* (E35M62-1, E33M61-1) and *Scmv2* (E33M61-2, E33M52, E38M51, E82M57, E84M59, E93M53) were identified in a BSA employing four DNA samples: both parental lines FAP1360A (resistant parent) and F7 (susceptible parent), as well as a resistant and a susceptible bulk (Xu et al. 1999). AFLP markers were named according to the standard list for AFLP primer nomenclature (Keygene, The Netherlands, http://wheat.pw.usda.gov/ggpages/keygene/AFLPs.html). AFLP primer pairs corresponding to the tightly linked AFLP markers were used to re-amplify the linked AFLP markers from resistant parent FAP1360A. The resulting bands were excised from the dried gel with a sharp, clean razor blade. The sliced DNA-containing gel was transferred into an Eppendorf tube, eluted twice with 200 µl TE (1 h each) and once with 200 µl ddH_2O (2 h). The eluted gel was then mixed with 50 µl ddH_2O and kept on boiling water for 5 min to release the DNA from the gel. After the gel debris was spun down, the DNA-containing supernatant was transferred into a new Eppendorf tube and used as template for the subsequent amplification.

For a given linked AFLP marker, the corresponding primer pair and the same reaction conditions as for the main amplification of AFLPs (Vos et al. 1995) were employed to re-amplify the isolated AFLP marker bands. Re-amplification products were excised from an agarose gel, extracted with Nucleospin Kit (Macherey & Nagel) and blunt-end cloned into the pBluescript vector.

Conversion of AFLP markers

After cloning, five white colonies from each transformation event were selected. Respective inserts were sequenced using the ALF-Express automated sequencer (Amersham Pharmacia, Freiburg). Sequencing reaction conditions were chosen following the manufacturer's (Amersham Pharmacia) suggestions, and the DNA sequences were analysed using the ALIGNPLUS 2.0 software package (http://www.scied.com/ses_alim.htm). If the sequencing of these first five clones showed identity for at least three of the five clones, new primers internal to the AFLP selective primers were designed using the PRIMER1.02 programme (http://www.scied.com/ses_pd4.htm) (Table 1). Otherwise, additional five white clones were sequenced to receive a majority of identical sequences for one genotype. The internal primers synthesized for fragments corresponding to the AFLP markers were employed to amplify fragments from the inbred lines F7 and FAP1360A, which represent the parent lines of the mapping population for BSA. Internal primers of the three AFLP markers E33M61-1, E33M61-2 and E35M62-1 were additionally employed on the four inbred lines D21, D32 (SCMV resistant), and D145, D408 (susceptible) in order to evaluate the relationship between polymorphisms and SCMV resistance. The extension "STS" was added to the names of the AFLP marker after synthesizing the internal primers in order to distinguish AFLP markers and converted markers.

Sequenced tagged site (STS) markers that differed in length after amplification were used immediately as indel (insertion/deletion) markers. In the case of an identical sequence length, enzyme recognition sites were identified using the CLONE manager software package (http://www.scied.com/ses_cm6.htm). Sequence regions displaying single nucleotide differences in restriction enzyme recognition sites between parent lines of mapping populations were used to identify CAPS markers, which were separated on a 3% MetaPhor agarose gel in 0.5× TBE buffer.

Linkage and statistical analyses

Based on the segregation data, the STS markers were mapped to previously constructed genetic linkage maps (Xia et al. 1999; Xu et al. 1999). Marker orders and map distances for population D32 × D145 were calculated with MAPMAKER 3.0B (Lander et al. 1987) using a LOD threshold of 3.0 and the mapping function of Kosambi (1944). Marker orders and genetic distances for population FAP1360A × F7 were calculated with CRIMAP 2.4 (Green et al. 1990) taking into account the meiotic interdependence of progenies and ancestors within a population of BC individuals from different generations.

Table 1 STS marker development in maize: detailed information on eight STS markers converted from AFLPs that are closely linked to resistance genes *Scmv1* (chromosome 6) and *Scmv2* (chromosome 3)

Marker locus	Chromosome	STS forward primer (5′ → 3′) STS reverse primer (5′ → 3′)	Sequence length after amplification with STS primers (bp)	Number of SNPs[a]	Marker type
		Primers analysed with inbred F7 and FAP1360A			
E33M52STS	3	CCATATCGTGTTGAGAAGGC CCACTCAATGCGGTGTCTAT	173	1	–
E38M51STS	3	CACCAAGAAGGTTTGGATCC GCGTACCAATTCACTAACCG	146	–	–
E82M57STS	3	AACCTCCTAGCGTCATGTAG AGTCCTGAGTAACGGATCC	166	–	–
E84M59STS	3	AACAACAGTTACCAGGCCAG CTTCAGATTCTCCCGAACCA	168	2	–
E93M53STS	3	GCTTGCCAATTCTGCATGCA	203	–	–
		Primers analysed with inbred lines F7, FAP1360A D21, D32, D145 and D408			
E35M62-1STS	6	GAGTCCTGAGTAACCGCCTA CTTCATGCCTCTCGTCG	152; 160	7	Indel
E33M61-1STS	6	ACTGCTTAGTCCTCGACAGA CGTACCAATTCAAGAGCGAC	195	–	–
E33M61-2STS	3	TCTTGTGCAACTACGACACC GATGATGGCATTGTCGAGGA	152	8	CAPS

[a] Identified between pairs of inbred lines

Results

The polymorphic markers linked to SCMV resistance, obtained after AFLP analysis, were in the range of 200 bp to 300 bp. All sequenced AFLP fragments contained the *Eco*RI adapter on the one end and the *Mse*I adapter on the other end. Five to ten clones obtained from inbred line FAP1360A were used to design primers for PCR amplification of genomic DNA (Table 1). Following amplification using these PCR primers, no difference in sequence length was identified for any of the markers between inbred lines F7 and FAP1360A, with sequence lengths ranging from 146 bp for marker E38M51STS to 203 bp for marker E93M53STS. For the additionally analysed inbred lines (D21, D32, D145 and D408), markers E33M61-1STS and E33M61-2STS showed identical sequence length for all six inbred lines (195 bp and 152 bp, respectively). A 152-bp sequence was identified with marker E35M62-1STS for all of the inbred lines D32, D21 and D408 except D145 (160 bp). This polymorphism of 8 bp between the parents of cross D32 × D145 could be easily detected on a 3% MetaPhor agarose gel (FMC) (Fig. 1). Genetic mapping was performed using 87 $F_{3:2}$ families of cross D32 × D145, which located E35M62-1STS on maize chromosome 6S between markers phi075 and phi077 within the *Scmv1* QTL region previously identified by Xia et al. (1999).

Six out of the eight markers showed identical sequences among the clones within each inbred line. In contrast, markers E33M61-1STS and E84M59STS resulted in single nucleotide differences between the clones within each inbred line. Single nucleotide polymorphisms (SNPs) between inbred lines F7 and FAP1360A were found for the four markers E33M61-2STS, E35M62-1STS, E33M52STS and E84M59STS. Recognition sites for restriction enzymes could be found only for marker E33M61-2STS, resulting in a different number of recognition sites for the restriction enzyme *Mnl*I (Fig. 2). For marker E33M61-2STS, *Mnl*I cuts the fragments of FAP1360A (Fig. 2) and D408 four times. The fragments of inbred lines F7, D21, D32 and D145 were cut only three times with *Mnl*I. Therefore, E33M61-2STS could be used as a CAPS marker (Fig. 3). Mapping of E33M61-2STS with the BC_5 mapping population (FAP1360A × F7) confirmed the same segregation pattern with its corresponding AFLP marker E33M61-2 and its location 7.3 cM above *Scmv*2.

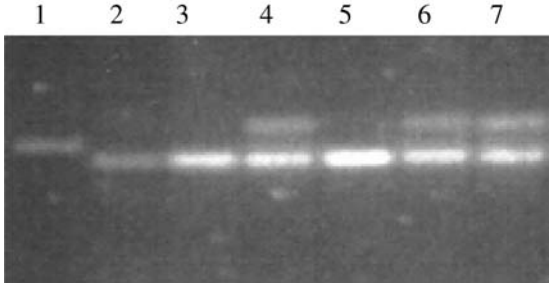

Fig. 1 Polymorphism in population D32 × D145 after conversion of AFLP primer E35M62-1 to the indel marker E35M62-1STS on a 1.5% agarose gel. *Lanes: 1* Susceptible parent D145, *2* resistant parent D32, *3–7* genotypes of the mapping population (*3*, *5* homozygous; *4*, *6*, *7* heterozygous band pattern)

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F7          (   1)   GATGATGGCATTGTCGAGGATGAAGTCCCTGTGGCTCGATGAAGATGTCG
FAP1360A    (   1)   GATGATGGCATTGTCGAGGATGAAGTCCCTGTGGCTCGATGAAGAgGTCG

F7          (  51)   GAGAAGGATTTCCAGCAACGTGCCCACGAGCTTGTGGGACATGTCCATGG
FAP1360A    (  51)   GAGAAGGATTTCCAGCAACGTGCCCACGAGCTTGTGGGACATGTCCATGG

F7          ( 101)   AGGCAAGTTGTCCTACTATTCCACCCAGGTCGTTCCAGCGAACCAGGTGG
FAP1360A    ( 101)   AGGCAAGTTGTCCTgCTATTCCACCCAGGTCGcTCCAaCGAACCAGGTGG

F7          ( 151)   CCTTGCCGCCAGAAAGTCATGGTGAGGGTGTCGTAGTTGCACAA
FAP1360A    ( 151)   CCTTGCCGCCAGAAAGTCATGGTGAGGGTGTCGTAGTTcCACAA
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Fig. 2 Recognition sites for restriction enzyme *Mnl*I in parents F7 (susceptible) and FAP1360A (resistant) after amplification with CAPS marker E33M61-2STS

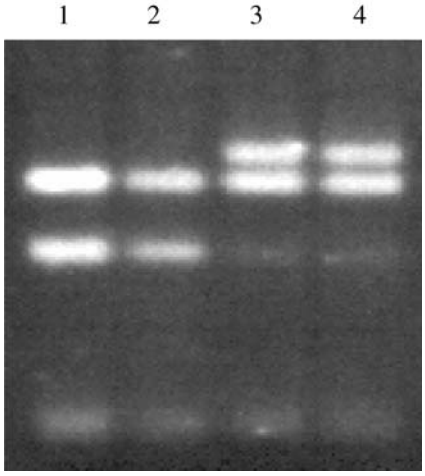

Fig. 3 CAPS marker E33M61-2STS digested with the restriction enzyme *Mnl*I corresponding to AFLP marker E33M61-2. *Lanes 1, 2* Resistant parent FAP1360A, *3*, *4* susceptible parent F7

Four out of the eight investigated markers revealed no SNPs after pairwise comparison of the inbreds. Although only half of the analysed markers showed between one and eight SNPs per inbred pair, 2.1 SNPs were found per inbred pair on average, resulting in one SNP per 71 bp. Analyses for stop codons identified continuous open reading frames for none of the sequences.

Discussion

In combination with BSA, AFLPs proved to be highly efficient for finding tightly linked molecular markers to the SCMV resistance genes *Scmv1* and *Scmv2* (Xu et al. 1999). However, AFLP markers are too costly and laborious for high-throughput monitoring of large numbers of genotypes. Hence, the conversion of AFLP markers closely linked to resistance genes is an important step to implement useful markers for MAS and map-based cloning, both of which require large population sizes of thousands of individuals. Marker conversion requires the characterization of the linked marker sequences and the design of locus-specific primers (Paran and Michelmore 1993). Reports on successful AFLP marker conversion are lacking so far in maize. With a size of 500–1500 bp, RAPD fragments are easier to convert to either sequence-characterised amplified region (SCAR) or CAPS markers than AFLP markers (Barret et al. 1998). Although there are doubts in converting short fragments like AFLPs (Negi et al. 2000), we were able to convert two short AFLP bands (150–300 bp) into PCR-based indel and CAPS markers without using methods like inverse PCR or chromosome walking. In contrast to DeJong et al. (1997) and Negi et al. (2000), who applied either inverse PCR or PCR walking to isolate the flanking regions for conversion of indel markers, we found with E35M62-1STS a polymorphism that could be used directly as an indel marker in populations generated from cross of D32 and D145.

After sequencing five to ten clones for each STS marker, we obtained six markers with identical sequences over most of the clones originating from one inbred. For markers E33M61-1STS and E84M53STS, SNPs were also found within all inbred lines. In total, 8 out of 28 inbreds investigated with the eight markers revealed single nucleotide changes within the inbred lines. The small number of published reports on AFLP marker conversion might be due to similar findings. Because identical sequences for the several clones of one inbred line were found technical problems in sequencing could be ruled out. The probability of residual heterozygosity for the inbred lines is below 0.025% because they were self-fertilized for more than 12 generations. Taking into account that the independent inbred lines revealed the same SNP within these inbreds in every case, it seems very unlikely that these polymorphisms were caused by residual heterozygosity. If we accept, the hypothesis that maize is an ancient tetraploid species (Gaut and Doebley 1997), the whole region harbouring the SCMV resistance gene might be duplicated, even though located at different regions of the genome. Under these conditions the segregation ratio would shift from 1r:3s (1 resistant to 3 susceptible) under a two dominant gene model for BC plants to 1r:7s under a three gene model. Although Xu et al. (1999) found a better fit with a three dominant gene model for the segregation within 20 $BC_{4:5}$ families of population F7 × FAP1360A segregating for SCMV resistance, the presence of additional SCMV resistance genes beside those on chromosomes 3 and 6 could not be confirmed for population F7 × FAP1360A. In contrast, mapping of the original AFLP markers did locate markers E33M61-1STS and E84M59STS exclusively to chromosomes 6 and 3, respectively.

Clusters of resistance genes originating from duplication during the evolution of maize may have led to slight sequence differences of paralogs, which may differ only in single nucleotides but not in total length. In mapping BAC clones of lettuce in order to analyze resistance gene clusters, Meyers et al. (1998) identified duplicates of AFLP markers in the same chromosome region. Genes conferring resistance to different pathogens are often clustered in the same chromosome region in the maize genome (McMullen and Simcox 1995). The fact that the AFLP markers corresponding to the converted STS markers mapped in the same regions previously reported to harbour clusters of resistance genes (McMullen et al. 1995) allows the assumption that the different marker sequences found within one inbred are linked to different resistance genes in the same chromosome region. The closer a marker is linked to a specific resistance gene, the higher might be the probability of being duplicated with the resistance gene during evolution. Hence, the occurrence of different sequences within one inbred line that map to the same chromosome region seems to be possible due to clustering. However, none of the sequenced AFLP fragments revealed any similarity to sequences known to be conserved within resistance genes.

The development of markers that can be easily handled is a prerequisite to the screening of large populations in order to clone the resistance genes *Scmv1* and *Scmv2*. The converted CAPS and indel markers will be useful to identify recombination events close to *Scmv1* and *Scmv2*. So far, it is unknown whether resistance genes cluster due to linkage or whether some of them are identical and display pleiotropy. In support of the existence of closely linked but different major resistance genes, Lübberstedt et al. (1999) found some susceptible plants in an allelism test between the three European dent inbreds D21, D32 and FAP1360A. The converted, closely linked markers identified in the present study could be used as probes for BAC screening in order to solve the question of whether the *Scmv1* and the *Scmv2* regions each harbour only a single locus or clusters of resistance loci.

Rafalski et al. (2001) analysed random cDNA clones in a collection of over 30 maize lines representative for the North American corn germplasm. Their analysis was restricted to coding regions. Sequence alignment revealed one SNP per 70 bp among the 30 lines. The authors emphasized that pairwise comparison between any two lines reveals a lower degree of polymorphism. In similar experiments, Useche et al. (2001) detected one SNP per 49 bp, although in non-coding regions. The low number of one SNP per 71 bp found in our study might be due to the pairwise sequence comparison in contrast to the sequence alignment of a large number of inbreds analysed by the previous authors. Taking into account that only four out of the eight converted AFLP markers showed polymorphism between inbred lines, it seems very likely that extending the fragment size by inverse PCR would increase the number of polymorphic STS-primers.

The CAPS marker E33M61-2STS turned out to be dominant in the mapping population. The polymorphism resulted in the presence of an additional band in the resistant parent FAP1360A that was absent in the susceptible parent F7 (Fig. 3). As in this mapping population the individuals were either homozygous for the susceptible parent allele or heterozygous, mapping with our mapping population of 27 resistant BC_5 individuals was not affected. However, even in this dominant case, CAPS markers are easier to apply than the original AFLP markers. In contrast to the AFLP markers, the converted markers do not require purified, high-molecular-weight DNA. Consequently, the application of simple STS markers enables a faster DNA isolation for a high number of individuals required for fine mapping. Additionally, the polymorphisms revealed by STS markers could be separated by an agarose gel, where no radioactivity is required to visualize the results. Compared to the analyses of AFLP markers in which polyacrylamide gels and radioactivity are used, the application of STS markers can reduce costs to about 20%.

The quality of a marker used for MAS depends on its predictive and/or diagnostic value (Borchardt and Weissleder 2000). Whereas the predictive value of a marker is determined by the inheritance of the marker and the linkage between marker and trait, the diagnostic value can be measured as the frequency of the desired linkage phase between marker and trait. Taking into account that resistant individuals of different populations harbour different resistance alleles of the same gene, cosegregation of these markers with the resistance trait in each population (F7 × FAP1360A, D32 × D145, D21 × D408) is not consequently preconditioned. By analysing inbred lines showing resistance, partial resistance and susceptibility to SCMV, Xu et al. (2000) suggested a single common ancestor for the resistance gene *Scmv1*. In the present study, no marker allele identical for all resistant or susceptible genotypes was identified. Therefore, the diagnostic value of these markers seems to be low. In the case of a low diagnostic value, the allelic phase of a marker has to be checked in each cross before it can be used in MAS (Borchardt and Weissleder 2000). A reason for the lack of resistance-allele-specific, cosegregating markers could be the presence of more than one SCMV resistance gene in the *Scmv1* region. Field experiments, BSA (Xu et al. 1999), and QTL analyses (Xia et al. 1999; Dussle et al. 2000) did not preclude the presence of more than one gene in the *Scmv1* region. Since different ancestors were expected for *Scmv2* (Dussle et al. 2000; Xu et al. 2000) and, therefore, different SCMV resistance genes within the *Scmv2* region, it was not possible to develop one single resistance-allele-specific marker for *Scmv2*.

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